December 2008

To the Graduate Council:

We are submitting a thesis written by Meagan A. Binkley entitled "The Phylogeography of North American Chestnuts and Chinquapins (*Castanea* Mill., Fagaceae)." We have examined the final copy of this thesis and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science with a major in Environmental Science.

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Dr. J. Hill Craddock, Co-chairperson Dr. Joey Shaw, Co-chairperson

I have read this thesis and
recommend its acceptance.

_____

Dr. Stylianos Chatzimanolis

Accepted for the
Graduate Council:

_____

Dr. Stephanie Bellar
Dean of the Graduate School

The Phylogeography of North American Chestnuts and Chinquapins (*Castanea* Mill., Fagaceae)

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee at Chattanooga

Meagan A. Binkley

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Abstract

North American *Castanea* Mill. (Fagaceae) consists of three morphologically variable species: *Castanea dentata*, *Castanea pumila* and *Castanea ozarkensis*. Taxonomy of these species has been complicated by intermediate morphology, similarity in growth habit, and putative naturally occurring hybridization where species' ranges overlap in the southeast. The main goals of the present study were to: 1) determine if genetics reflect the morphological variation observed in southeastern populations of North American *Castanea*, 2) explore the extent of cpDNA haplotype sharing among these taxa, and 3) map haplotype distribution in relation to morphotaxa. Finally, I wanted to use the information obtained from these analyses to gain insight into southeastern populations, where intermediate morphologies and putative hybridization are confounding taxonomy. I sequenced the *trn*V-*ndh*C intergenic spacer region (~380 bp) of the chloroplast for 233 *Castanea* accessions collected throughout the range of the genus, with a focus on southern Appalachian populations from Georgia, Tennessee and North Carolina. I identified four main chloroplast haplotypes and found that for three of these haplotypes, leaf morphology is a reliable indicator of haplotype identity. I found that the fourth haplotype is shared among accessions of *C. dentata* and *C. pumila*, and is also found in trees with intermediate morphology. The geographic mapping of these haplotypes revealed that each haplotype is found in a separate geographic range, allowing for the comparison of current distributions with locations of possible glacial refugia. Although the precise genetic basis of the confounding morphology in southeastern populations of North American *Castanea* is still uncertain, an intricate biogeographical history combined with the tendency of taxa within this genus to share haplotypes in sympatric areas could explain much of the morphological complexity of *Castanea*.

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Nomenclature

cm	centimeter
mmol	millimole
g	gram
L	liter
M	meter
$MgCl_2$	magnesium chloride
ng	nanogram
µL	microliter
µmol	micromole

Abbreviations

ABI	Applied Biosystems, Incorporated
ACCF	American Chestnut Cooperator's Foundation
AFLP	amplified fragment length polymorphism
bp	base pair
CI	consistency index
cp	chloroplast
cpDNA	chloroplast DNA
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
indel	insertion/deletion
LSC	large single copy
MCMC	Markov Chain Monte Carlo
mya	million years ago
PAUP	Phylogenetic Analysis Using Parsimony
PCR	polymerase chain reaction
RAPD	random amplification of polymorphic DNA
RFLP	restriction fragment length polymorphism
RI	retention index
TACF	The American Chestnut Foundation
TBR	tree bisection reconnection
TCS	Templeton, Crandall and Sing
UCHT	University of Tennessee-Chattanooga Herbarium
USGS	United States Geological Society
ya	years ago

Chapter 1

Overview of the North American Chestnuts and Chinquapins (*Castanea* Mill., Fagaceae)

Castanea Miller is a genus of chestnuts and chinquapins within the family Fagaceae, which also includes *Fagus* L. (beeches), *Quercus* L. (oaks), *Lithocarpus* Bl. (tanoaks), *Castanopsis* Spach. (Asian chinquapins), *Chrysolepsis* Hjelmquist (golden chinquapins), *Colombobalanus* (Lozano, Hdz-C. & Henao) Nixon & Crepet, *Formanodendron* (Camus) Nixon & Crepet, and *Trigonobalanus* Forman. Approximately 1000 species belong to this family, and the majority is distributed among the forests of the North Temperate Zone (Manos et al. 2001). Although past estimates of the number of *Castanea* species are as high as thirteen (Camus 1929), the current number recognized is more conservative: eight species historically divided among three sections. Section *Eucastanon*, characterized by 3 nuts per bur, is found in China (*C. mollissima* BL. and *C. seguinii* Dode.), Japan (*C. crenata* Sieb. & Zucc.), North America (*C. dentata* (Marsh.) Brokh), and Europe (*C. sativa* Mill.) (Jaynes 1975). Two sections, characterized by one nut per bur, contain chinquapins: *Hypocastanon* is found in China (*C. henryi* [(Skan) Rehder & Wilson]) and *Balanocastanon* is found in the southeastern United States (*C. pumila* Mill.) and in the Ozark Mountains (*C. ozarkensis* Ashe) (Nixon 1997).

As a member of the arcto-Tertiary geoflora, *Castanea* has a disjunct distribution between eastern Asia and eastern North America that is a relict of the more geographically widespread Tertiary temperate forests (Wen 1999). It is likely that many now extinct *Castanea* species once had broader distribution across the Northern Hemisphere during this time period. Current patterns of disjunction were probably

established within *Castanea* between 10-15 million years ago (Dane et al. 2003). Species in these two areas have similar distribution patterns, as the ranges of geographically widespread species (*C. mollissima* and *C. dentata*) overlap to varying extent with the ranges of more geographically restricted species (*C. pumila*, *C. henryi* and *C. seguinii*) (Lang et al. 2007).

Castanea dentata was once one of the most dominant timber and nut-producing trees in eastern North America (Craddock 2006). Approximately four billion *C. dentata* trees populated the forests of the eastern United States; one in every four trees in the central Appalachian forest was a chestnut (Saucier 1973). Historically, *C. dentata* ranged over 200 million acres (MacDonald 1978; Brown and Davis 1995) of the eastern United States, from Maine to Georgia and west to Mississippi, including West Virginia, Kentucky, Tennessee, Indiana, southern Michigan, Arkansas, and Missouri (Nixon 1997). American chestnut trees comprised approximately twenty percent of the Appalachian forest (Kulman 1978); however, in the southern Appalachians, the density of *C. dentata* reached 70-80% (Sherald 2006). In several places in western North Carolina, American chestnut grew in nearly pure stands up to 100 acres in size (Ashe 1912).

C. dentata grew up to 37 meters tall with a diameter of two meters and was highly valued as a timber tree because of its straight, branchless, decay-resistant boles up to fifteen meters long. The fruit of *C. dentata* was a significant food source for both humans and wildlife and served as a cash crop for many Appalachian families (Myers et al. 2004). The largest chestnut trees could produce over ten bushels of nuts, making *C. dentata* one of the most important mast trees for wildlife in the eastern United States (Davis 2006), providing a major food source for a diverse array of animals such as

squirrel, wild turkey, grouse, wild turkey, raccoon, white-tailed deer and black bear. Humans had extensive culinary use for chestnuts and depended on the mast crop as an inexpensive way to fatten hogs. American chestnuts were also a highly valued building material, ideal for projects requiring long-lasting and rot resistant wood, such as roof shingles, telephone poles, ship masts, and railroad ties. A secondary use of chestnut was as a source of tannic acid, which the leather industry obtained from chestnut bark and rough chestnut cordwood (Davis 2006). Thus, *C. dentata* filled an important socioeconomic and ecological niche as one of the most important timber and mast trees in the eastern United States, until the introduction of a fungal blight in the early 20th century nearly annihilated the species (Roane et al. 1986).

In 1904, Herman Merkel, a forester at the New York Zoological Gardens, discovered a fungus infecting American chestnuts. This fungus, *Cryphonectria parasitica* (Murr.) Barr, was new to science and eventually came to be known as the causal agent of chestnut blight (Merkel 1906). *Cryphonectria parasitica* was brought to the United States on Asian *Castanea* nursery stock imported to New York for the purpose of breeding with native chestnuts to make a hybrid nut that would combine the size of Asian fruits with the sweetness prized in the American chestnuts (Anagnostakis 1987). The first scientific publication about the fungus, first known as *Endothia parasitica*, (Murrill) P.J. & H.W. Anderson, was published in 1908 (Murrill 1908). *Cryphonectria parasitica* infects American chestnut trees through cracks in the bark (Kuhlman 1978). Fine threads of mycelia enter into the inner bark where they kill the cambium layer, causing sunken, necrotic cankers that expand until they encircle the infected tree trunk or branch and kill all distal foliage. Cankers secrete masses of conidia that are transferred to other trees via

insects, birds, and mammals, including human beings (Anagnostakis and Hillman 1992). Wind also can transfer the ascospores to nearby American chestnut trees (Anagnostakis 1987). *Castanea* species vary in their level of resistance to *C. parasitica*. Asian species appear to be fairly blight-resistant, whereas North American and European *Castanea* taxa are known to be highly vulnerable to chestnut blight (Jaynes 1975).

The devastation of the American chestnut by the chestnut blight was perhaps one of the greatest catastrophes in the recent history of forests (MacDonald 1978). By 1912, all of the chestnut trees in New York were dead, and the disease continued southward at a rate of up to 50 miles per year (Davis 2006). As chestnut blight continued its deadly spread, the majority of research efforts shifted focus from describing the biology of *C. parasitica* to attempting to breed blight resistant chestnut trees (van Fleet 1914). By the 1930's, the blight pandemic had spread to Europe and was found on *C. sativa*. By 1940, there were few American chestnuts in the entire Appalachian region that remained alive and without evidence of serious infection by blight (Davis 2000). All attempts of controlling the fungus through pruning, cutting, and spraying failed. The blight spread rapidly, and within fifty years, the main stems of American chestnuts had largely died throughout the eastern deciduous forests (Beattie and Diller 1954; Roane et al. 1986; Anagnostakis 1987; Freinkel 2007). By 1950, the chestnut blight had decimated nearly all of the large stems of American chestnut trees within their native range (Hebard 2006). While the loss of the American chestnut affected wildlife populations and altered forest ecology, perhaps the loss was most noticeable in the effect it had on Appalachian culture. Indeed, Davis (2006) proposed that the downfall and ultimate loss of Appalachian subsistence culture can be attributed in part to the loss of the American chestnut to the

chestnut blight. Since the introduction of chestnut blight, American chestnuts have been reduced largely to chestnut sprouts or remnant populations of understory shrubs with occasional medium-sized trees observed; however, few of these individuals live to reach the fruiting stage, which precludes nearly all naturally-occurring sexual reproduction in the wild (Paillet 2003).

Throughout the decline of American chestnut, there have been many efforts to restore *C. dentata* to its previous ecological and economic roles and prevent the extinction of the species. By the 1930's, the gravity of the chestnut blight and the apparent doom of American chestnut were recognized and programs dedicated to breeding a blight-resistant chestnut tree began (Hebard 2006); however, none of these programs was designed to control blight in eastern forests (MacDonald and Double 2006). By the 1960's many of these attempts ceased when people began to realize that there had been little success in breeding a tree that contained the blight resistance of Asian chestnut trees with the stature of American chestnut trees (Jaynes 1994).

In 1961, what later came to be recognized as viruses (Hillman et al. 2000), were discovered infecting *C. parasitica* in *C. sativa* from Italy (Grente 1961). These viruses weakened the virulence of chestnut blight enough that infected strains of *C. parasitica* were unable to kill European chestnut. This discovery of debilitated strains of *C. parasitica* ('hypovirulent' strains, as coined by Grente), and the fact that this weakened state could be transmitted to virulent strains (Grente 1965) led to the hope that chestnut blight infections in American chestnut trees could be biologically controlled. Although these efforts continue today, this method of blight control has never been as successful with N. America as it was in Europe (Anagnostakis 1990).

The contributions of early chestnut pioneers such as the United States Department of Agriculture, chestnut blight commissions, and experimental research stations laid the groundwork for the two organizations currently working to breed a blight-resistant chestnut: The American Chestnut Foundation (TACF) and the American Chestnut Cooperators' Foundation (ACCF). In 1983, the American Chestnut Foundation was founded as a not-for-profit organization to help fund research based on the hypothesis that the blight resistance of Asian chestnut trees such as *C. mollissima* could be backcrossed into American chestnut, a classical approach to plant breeding that had not been utilized in the prior efforts to breed a blight-resistant, timber-type chestnut (Burnham et al. 1986). TACF heads an extensive restoration project with the goal of restoring the American chestnut to the forests of the Eastern United States. In 1984, ACCF was begun as a non-profit scientific and educational foundation with the parallel goal of restoring American chestnut.

Although these two groups have similar goals, they employ two separate strategies. The focus of ACCF is a breeding program using all North American genes to derive native North American gene combinations that confer blight resistance by interbreeding trees of low resistance to accumulate genes, cloning resistant scions on existing sprouts, and selecting and managing sites optimum for growth (Griffin 2000). In an attempt to combine the blight resistance of Asian chestnut trees with the lumber quality of American chestnut, TACF is focused on a backcross breeding method in which surviving *C. dentata* trees are bred to Chinese chestnut, *C. mollissima*, and Japanese chestnut, *C. crenata*, with the ultimate goal of creating a blight-resistant chestnut hybrid that can be a useful canopy tree. As of 2006, TACF had recovered highly blight-resistant

chestnut trees after two cycles of backcrossing Chinese blight resistance into American chestnut trees, and they had high hopes that the third cycle of backcrossing would result in highly resistant hybrid chestnut trees comparable to American chestnuts in look and growth. In 2006, TACF projected that by 2008, the progeny of these trees would be ready to be planted back into the forests and begin to fulfill their hopes of restoring the American chestnut trees to the forests of Appalachia (Hebard 2006). In fact, experimental plant of TACF B_3F_3 seedlings began in 2008 on National Forest property (Craddock, pers. comm.)

TACF is organized into state chapters. Each has its own breeding program to ensure that local genotypes are incorporated into regionally developed hybrids. Currently there are active chapters of TACF in Maine, Massachusetts, Connecticut, New York, Pennsylvania, Indiana, Kentucky, the Carolinas, Georgia, Alabama, and Tennessee (Craddock 2006). Recent efforts of the Alabama, Georgia, and Tennessee chapters to locate potential *C. dentata* to use for their breeding programs have been confounded by intermediate morphologies in southeastern *Castanea* populations where these taxa are difficult to identify in the absence of reproductive morphological characters, when species' calls must be based solely on leaf morphology. The trees in one such population in northwest Georgia, hereafter referred to as the 'Pocket' population, resemble *C. dentata* in having large, dentate, essentially glabrous leaves yet resemble *C. pumila* in floral and fruit morphology (single fruits per cupule) (J.H. Craddock, University of Tennessee at Chattanooga, pers. comm.) Confounding morphology is of particular interest to TACF's Georgia chapter, because *C. dentata* has limited distribution in this southern extent of its range, thus flowering trees are relatively rare. Confounding

taxonomy and near-impossible field identification of these southeastern populations with intermediate morphology such as the Pocket raise questions about the identity of these trees.

I began this course of study with a seemingly straightforward goal to determine how the Pocket population fits into the taxonomy of N. American *Castanea*. I hypothesized that the trees within the Pocket population were either: 1) a morphotype of *C. pumila,* 2) the naturally-occurring hybrid taxon, *C. X neglecta*, 3) man-made Asian-American *Castanea* hybrids, or 4) a disjunct population of *C. ozarkensis*. Perhaps the first hypothesis is the most parsimonious, as much taxonomic debate has centered on the classification of *C. pumila*, a widely discussed and highly variable taxon. Since Linnaeus's initial description of *Fagus pumila* (Linn.) in 1735, an additional 28 new taxa or combinations have been proposed for *C. pumila* (Johnson 1988). As this species currently encompasses various morphotypes and growth habits, it seems reasonable to predict that the intermediate morphologies seen in populations such as the Pocket might also be *C. pumila*. If this is the explanation for the identity of the Pocket trees, then I might expect them to have the same haplotype as *C. pumila*.

Castanea taxa appear have little to no reproductive barriers (Johnson 1988) and can hybridize (Rutter et al. 1990). Many botanists have noted the presence of putative hybrid populations of *C. dentata* X *C. pumila* resulting in *Castanea X neglecta*, Dode. in the southern Appalachian region (Small 1933; Radford et al. 1964; Wofford 1989; Weakley, in prep). *Castanea X neglecta* is believed to be widespread throughout the southern Appalachian Mountains and difficult to separate from *C. pumila* (Nixon 1997). The hypothesis that the Pocket trees might be *C. X neglecta*, results from the fact that

these trees have unusual leaf morphology and are located within the geographic region where this putative hybrid was historically purported to exist. If the Pocket trees are *C. X neglecta*, I would expect to see that the Pocket trees might contain the *C. dentata* haplotype, the *C. pumila* haplotype, or a combination of both since the chloroplast genome is nonrecombinant and uniparentally inherited.

Evidence exists for the third hypothesis that 'Pocket' trees could be an anthropogenic population of Asian-American chestnut hybrids. In the late 1930s through the mid-1940s, organizations such as the Civilian Conservation Corps planted man-made Asian-American hybrid trees in Floyd County, Georgia, to combat the loss of American chestnuts to chestnut blight (S. Anagnostakis, The Connecticut Agricultural Experiment Station, pers. comm.) In light of this evidence, combined with intriguing leaf morphology that could be construed to resemble that of *C. crenata*, it is also possible that the Pocket trees may be a remnant hybrid population left over from these early attempts to 'save' the American chestnut tree from extinction. If Pocket trees are Asian-American hybrids, I might anticipate that these trees would have an Asian chloroplast haplotype or North American haplotype.

Finally, there is the possibility that the Pocket trees are a disjunct population of *C. ozarkensis*. Although the current range of *C. ozarkensis* is restricted to the Ozark Mountains, *C. ozarkensis* is historically noted to have existed as far eastward as north-central Alabama (Johnson 1988); however the last sighting of any putative *C. ozarkensis* in that area dates back to the mid-1970's. As of the late 1980's, *C. ozarkensis* had apparently been extirpated from this area because of chestnut blight (Johnson 1988). The fact that this species was historically found in northern Alabama led to the hypothesis that

the Pocket trees might be *C. ozarkensis*. If this is the case, then I would expect for Pocket trees to have the same chloroplast haplotype as the Ozark chinquapins from the extant western populations.

To comprehensively address the aforementioned questions, we first screened the chloroplast genome to identify a DNA region that would provide adequate information to separate the currently recognized North American *Castanea* species. Once we established that the *trn*V-*ndh*C intergenic spacer region would provide enough genetic information to separate the three North American species (Kennedy 2008), I then employed a phylogeographic sampling scheme where samples of all recognized taxa were obtained from throughout the extent of their respective ranges.

List of References

Anagnostakis, S.L. 1987. Chestnut blight- the classical problem of an introduced pathogen. *Mycologia* 79(1): 23-37.

Anagnostakis, S.L. 1990. Improved chestnut tree condition maintained in two Connecticut plots after treatment with hypovirulent strains of the chestnut blight virus. *Forestry Science.* 36:113-124.

Anagnostakis, S.L. and B.I. Hillman 1992. History of chestnut breeding in the United States. P. 19-21. In Proceeding of the World Chestnut Industry Conference. Wallace, R.D. and L.G. Spinella, eds. Chestnut Marketing Association Press, Alachua, FL.

Ashe, W.W. 1912. Chestnut in Tennessee. *Tennessee Geological Survey Bulletin* 10-B: 35.

Beattie, R.K., and J.D. Diller.1954. Fifty years of chestnut blight in America. *Journal of Forestry* 52(5): 323-329.

Brown, M. and D. Davis. 1992. Trail History Notebook. Great Smoky Mountains Natural History Association, Gatlinburg, Tennessee. 134 p.

Burnham, C.R., P.A. Rutter, and D.W. French. 1986. Breeding blight-resistant chestnuts. P. 347-397. In *Plant Breeding Reviews* 4. AVI Publishing Co. Westport, Connecticut.

Camus, A. 1929. The Chestnuts: Monograph of *Castanea* and *Castanopsis*. Paul Lechevalier. Paris.

Craddock, J.H. 2006. Chestnut breeding in the United States. P. 109-128 in Proceedings of the International Symposium for the 50th Anniversary of Korea Forest Genetics Researches & the 20th Anniversary of the late Dr. S.K. Hyun. June 15-16, 2006. Forest Seed Research Center (Suanbo, Korea).

Dane, F., P. Lang, H. Huang, and Y. Fu. 2003. Intercontinental genetic divergence of *Castanea* species in eastern Asia and eastern North America. *Heredity* 91: 314-321.

Davis, D. E. 2000. Where there are mountains: An environmental history of the southern Appalachians. University of Georgia Press, Athens, GA. 320 p.

Davis, D.E. 2006. Historical significance of American chestnut to Appalachian culture and ecology, *in* Steiner, K.C., and J.E. Carlson, eds. 2006. Restoration of American chestnut to Forest Lands- Proceedings of a Conference and Workshop. May 4-6, 2004, The North Carolina Arboretum. Natural Resources Report NPS/NCR/CUE/NRR- 2006/001, National Park Service. Washington, DC.

Freinkel, S. 2007. American Chestnut: The Life, Death, and Rebirth of a Perfect Tree. University of California Press.

Grente, J. 1961. Observations sur le comportement des plants de chataignier après inoculation de *l'Endothia parasitica*. *Ann. Epiphytes* 12:65-70.

Grente, J. 1965. Les formes hypovirulentes *d'Endothia parasitica* et les espoirs de lutte contre le chancre du chantaingnier. *C. R. Acad. Agric. France* 51: 1033-1037.

Griffin, G.A. 2000. Blight control and restoration of the American chestnut. *Journal of Forestry* 98 (2):22-27.

Hebard 2006.The backcross breeding program of the American Chestnut Foundation. P. 61-77, *in* Steiner, K.C., and J.E. Carlson, eds. 2006. Restoration of American chestnut to Forest Lands- Proceedings of a Conference and Workshop. May 4-6, 2004, The North Carolina Arboretum. Natural Resources Report NPS/NCR/CUE/NRR- 2006/001, National Park Service. Washington, DC.

Hillman, B.I., D.W. Fulbright, D.L. Nuss, and N.K. Van Alfen. 2000. Hypoviridae. P. 515-520 in Virus Taxonomy: Seventh Report of the International Committee for the Taxonomy of Viruses, van Regenmortel et al., eds. Academic Press, New York.

Jaynes, R.A. 1975. Chestnut. P. 503-590 *in* Advances in Fruit Breeding. Janisck, J. and J. Moore, eds. Purdue University Press, West Lafayette, IN.

Jaynes, R.A. 1994. Reflections. P. 45-46 *in* Proceedings of the International Chestnut Conference, Double, M.L. and W.L. MacDonald, eds. West Virginia University Press, Morgantown.

Johnson, G.P. 1988. Revision of *Castanea* Sect. Balanocastanon (Fagaceae). *Journal of the Arnold Arboretum* 69: 25-49.

Kennedy, S. "Chloroplast DNA analysis of putative chestnut chinquapin hybrids" (undergraduate thesis, University of Tennessee-Chattanooga, 2008), 20-22.

Kulman, E.G. 1978. The devastation of American chestnut by blight. P. 1-3 *in* Proc. Of the American chestnut symposium, MacDonald et al., eds. West Virginia University Press, Morgantown, WV.

Lang, P., F. Dane, T.L. Kubisiak, H. Huang. 2007. Molecular evidence for an Asian origin and a unique westward migration of species in the genus *Castanea* via Europe to North America. *Molecular Phylogenetics and Evolution* 43, 49-50.

MacDonald, W.L. 1978. Forward. P. v *in* Proc. Of the American chestnut symposium, MacDonald et al. eds. West Virginia University Press, Morgantown, WV.

MacDonald, W.L. and M.L. Double. 2006. Hypovirulence: use and limitations as a chestnut blight biological control, *in* Steiner, K.C., and J.E. Carlson, eds. 2006. Restoration of American chestnut to Forest Lands- Proceedings of a Conference and Workshop. May 4-6, 2004, The North Carolina Arboretum. Natural Resources Report NPS/NCR/CUE/NRR- 2006/001, National Park Service. Washington, DC.

Manos, P.S., Zhe-Kun Zhou, and C. H. Cannon. 2001. Systematics of Fagaceae: phylogenetics tests of reproductive trait evolution. *International Journal of Plant Sciences* 162(6):1361-1379.

Merkel, H.W. 1906. A deadly fungus on the American chestnut. *Annals of the New York Academy of Sciences* 10:97-103.

Murrill, W.A. 1908. The spread of the chestnut disease. *Journal of the New York Botanical Garden* 9:23-30.

Myers, B.R., J.L. Walck, K.E. Blum. 2004. Vegetation change in a former chestnut stand on the Cumberland Plateau of Tennessee during an 80-year period (1921-2000). *Castanea* 69(2): 81–91.

Nixon, K. C. 1997. Flora of North America Editorial Committee, eds., Flora of North America North of Mexico, Vol 3. Oxford University Press, NY, pp. 436-437.

Paillet 2003. Chestnut: history and ecology of a transformed species. *Biogeography* 29: 1517-1530.

Radford, A.E., H.E. Ahles, and C. R. Bell. 1968. Manual of the Vascular Flora of the Carolinas. University of North Carolina Press, Chapel Hill, North Carolina.

Roane, M.K., J.G. Griffin, and J.R. Elkins. 1986. Chestnut Blight, Other *Endothia* Diseases, and the Genus *Endothia*. APS Press. St. Paul, Minnesota. 53 p.

Rutter, P.A., G. Miller, and J.A. Payne. 1990. Chestnut. In J.N. Moore, and J.R. Ballington, Jr., eds. Genetic resource for temperate fruit and nut crops, 761-788. The International Society for Horticultural Science, Wageningen, The Netherlands.

Saucier, J. 1973. American chestnut: an American wood. FS-230, USDA. Forest Service. Washington: U.S. Government Print Off.

Sherald, J.L. 2006. Introduction, in Steiner, K.C., and J.E. Carlson, eds. 2006. Restoration of American chestnut to Forest Lands- Proceedings of a Conference and Workshop. May 4-6, 2004, The North Carolina Arboretum. Natural Resources Report NPS/NCR/CUE/NRR- 2006/001, National Park Service. Washington, DC.

Small, J.K. 1933. Manual of the Southeastern Flora. Published by Author, New York, New York.

van Fleet, W. 1914. Chestnut breeding experience. *Journal of Heredity* 5: 19-25.

Weakley, A. S. in prep. Flora of the Carolinas, Virginia, Georgia, and Surrounding Areas.

Wen, J. 1999. Evolution of eastern Asian and eastern North American disjunct distributions in flowering plants. *Annual Review of Ecological Systems* 30:421-455.

Wofford, E.B. 1989. Guide to the vascular plants of the Blue Ridge. University of Georgia Press, Athens, GA.

Chapter II

The Phylogeography of North American Chestnuts and Chinquapins (*Castanea* Mill., Fagaceae)

This chapter is a version of a manuscript by the same name submitted for publication in *Systematic Botany* by Meagan Binkley, J. Hill Craddock and Joey Shaw.

Introduction

Morphology is a primary tool used to discern taxa (Krishnankutty and Chandrasekaran 2008); however, inter- and intra-specific morphological variability can lead to considerable confusion in discriminating species. Ascertaining taxonomy based on labile morphological characters proves difficult when these characters vary widely within individuals and across populations. Intermediate morphology between closely related sympatric species adds complexity, and hybridization may further complicate matters (Rossetto 2005). Integration of molecular and morphological data has proven useful for resolving systematic questions in taxonomically uncertain groups (Oja and Jaaska 1998; Furini and Wunder 2004; Les et al. 2005; Lohmann 2006; Valiejo-Roman et al. 2006). In particular, chloroplast DNA sequences have added greatly to the understanding of complicated evolutionary histories of plants. Many studies show varying levels of intraspecific cpDNA variation (Soltis et al. 1992; Sewell et al. 1996; Gonzales et al. 2008; Morris et al. 2008), and several recent studies have used the strategy of coupling relatively quickly evolving chloroplast DNA sequences with a phylogeographic sampling scheme (i.e., sampling multiple individuals per taxon) to illuminate complex evolutionary histories in plants (Shaw and Small 2005, Jakob and Blattner 2006, Naciri and Gaudeul 2007, Prentice et al. 2008). Many recent studies

(Dumolin-Lapegue et al. 1997; Comes and Abbott 1999; McKinnon et al. 1999, Golden and Bain 2000; Matos and Schaal 2000; McKinnon et al. 2001; Devos et al. 2003; Kanno et al. 2004; Jakob and Blattner 2006) have revealed intraspecific sharing of cpDNA haplotypes, indicating the importance of hybridization and introgression within closely related plant taxa.

North American *Castanea* taxa are taxonomically challenging. Detailed systematic work on N. American *Castanea* began in the early 1900s and taxonomists have split N. American *Castanea* into as few as two (Small 1903; Robinson and Fernald 1908; Sargent 1922; Johnson 1988; Wofford 1989; Gleason and Cronquist 1991; Jones 2005) and as many as seven (Small 1933) and thirteen (Camus 1929) species. Much of the taxonomic debate has centered on the classification of *C. pumila*, a widely discussed and highly variable taxon. Since Linnaeus's initial description of *Fagus pumila* (Linn.) in 1735, an additional 28 new taxa or combinations have been proposed for *C. pumila* (Johnson 1988). The overall trend has been to reduce the number of N. American *Castanea* taxa (Johnson 1988), and three species are currently recognized: American chestnut (*Castanea dentata*), Allegheny chinquapin (*Castanea pumila*), and Ozark chinquapin (*Castanea ozarkensis*). *Castanea dentata* ranges throughout much of the eastern United States, from Maine to Georgia and west to Mississippi, including West Virginia, Kentucky, Tennessee, Indiana, extreme southeastern Michigan, Arkansas, and Missouri (Nixon 1997). *Castanea pumila* is found in the eastern and southeastern United States, from Massachusetts to Florida and west to Arkansas. *Castanea ozarkensis* is restricted to the Ozark Plateau, though disjunct populations of putative C. ozarkensis were observed in northern Alabama through the mid-1970s (Johnson 1988).

Taxonomic designations for N. American *Castanea* have been based on leaf characters as well as fruit to cupule ratios (Nixon and Crepet 1989); however, many morphological characters are not discrete, as is evidenced by taxonomic instability in this genus. Nowhere is morphological ambiguity more apparent than in populations from the southern Appalachians, where the range of *C. pumila* overlaps with the southern range of *C. dentata*. Boundaries between the two species are difficult to establish due to intraspecific variation, interspecific similarities, and putative interspecific hybridization (Rehder 1940; Jaynes 1964; Johnson 1988).

Complicating species distinction, N. American *Castanea* taxa are highly susceptible to chestnut blight, caused by a fungus, the ascomycete *Cryphonectria parasitica* (Murr.) Barr., which prevents many individuals from maturing to the point of flower and fruit production; thus, in areas where their ranges overlap, we are often relegated to using leaf morphology to differentiate between *Castanea* species. Variability in growth habit further confounds taxonomy, as chestnut blight has reduced *C. dentata* from a large canopy tree to a multi-stemmed shrub or small tree (Nixon 1997), now similar in habit to *C. pumila* and *C. ozarkensis*. The intraspecific morphological variation of *C. pumila* is an additional difficulty; for example, *C. pumila* can exist as a soboliferous shrub (previously treated as *C. alnifolia* Nutt.), a shrub, or a small tree (Johnson 1988). Finally, *Castanea* taxa have little to no reproductive barriers (Johnson 1988) and are known to hybridize (Rutter et al. 1990) in the southern Appalachian region (Small 1933; Radford et al. 1964; Wofford 1989; Weakley 2005). *Castanea X neglecta* Dode. is widespread throughout the southern Appalachian Mountains and difficult to separate from *C. pumila* (Nixon 1997). Additional data are necessary to better resolve the

relationships between these closely related taxa.

Molecular phylogeographic research of tree species has been significantly aided by amplification of specific cpDNA loci with universal primers (Newton et al. 1999). The wide availability and ease of obtaining DNA sequence data has resulted in a move from high level phylogenetic studies to research that also examines intraspecific variation, especially as it relates to geography (Avise 2000). Chloroplast DNA molecular data have proven to be useful tools for illuminating haplotype sharing (McKinnon et al. 2001; Shaw and Small 2005), detecting recent hybridization (Daehler and Strong 1997), and elucidating past hybridization and cytoplasmic introgression when chloroplast capture results from continuous, unilateral backcrossing of hybrid progeny with a pure parent species (Avise 1994). Thus the chloroplast genome has potential for exploring the evolutionary history of N. American *Castanea* species and the complex morphology observed in these taxa. Molecular data can add support to morphological taxonomy and allow for more robust delineation of relationships (Makarevitch et al. 2003; Furini and Wunder 2004); however, molecular data may contradict morphological taxonomy (McKinnon et al. 1999; McKinnon et al. 2001; Shaw and Small 2005). Regardless of whether molecular data support or refute morphological taxonomy, cpDNA data often reveal underlying genetic structure opaque to studies based strictly on morphology.

Molecular tools have been used to progress our understanding of the genetic diversity and relationships of N. American *Castanea*. Kubisiak et al. (1997) assayed 241 polymorphic markers, including eight isozymes, 17 restriction fragment length polymorphisms (RFLPs), and 216 random amplified polymorphic DNAs (RAPDs) for 102 individuals of an F_2 family to map chestnut blight resistance in a three-generation *C.*

dentata X *C. mollissima* pedigree. They showed that certain molecular markers significantly associate with morphological features and supported the existence of three putative resistance loci. Huang et al. (1998) used allozyme and RAPD analyses to estimate genetic variation among 12 wild populations of American chestnut, revealing that *C. dentata* had higher genetic diversity in the southern populations and lower genetic diversity in northern populations; approximately 90% of the allozyme diversity existed within their defined populations. American chestnut had lower genetic diversity than congener species such as *C. mollisima* and *C. seguinii*, which may have been a factor that contributed to the demise of the species (Huang et al.1998). Dane et al. (2003) used allozyme analysis of 62 wild *Castanea* populations to investigate evolutionary relationships between N. American and eastern Asian chestnut species. They found that *C. dentata*, despite its wide geographic range, had the lowest genetic diversity, whereas *C. ozarkensis* had high genetic variability even though all individuals came from a limited area. In both American and Asian species, the more geographically restricted species such as the chinquapins had relatively higher genetic diversities. The Ozark chinquapin showed higher divergence from the Chinese chestnut species than did the American chestnut or Allegheny chinquapin. Fu and Dane (2003) examined allozyme variation in 12 *C. pumila* populations. They found widespread sharing of common alleles across populations, although no single population had all 18 alleles. Seventy percent of the overall allozyme variation occurred within *C. pumila* populations. *C. pumila* had high genetic diversity at the species level, similar to that of *C. ozarkensis*, and exhibited a much higher diversity than *C. dentata*. Unlike *C. dentata* (Dane et al. 2003), there was no discernable pattern of genetic differentiation across the geographic range of *C. pumila*.

Kubisiak and Roberds (2006) used 19 RAPD markers and six microsatellite markers to determine the genetic structure (variation in allele and haplotype frequency) of 993 *C. dentata* accessions from 18 populations to inform TACF's decisions on the necessary number of locations for its breeding program. They found that *C. dentata* had high levels of microsatellite and RAPD variability among populations. Interestingly, most of the genetic diversity and rare alleles were located in the southeastern portion of chestnut's range. Results from these studies imply that 95% of the neutral genetic variation with *C. dentata* can be obtained by sampling within any one population; however, in order to account for genetic differentiation of adaptive genes and gene complexes, Kubisiak and Roberds (2006) cautioned that many individuals (50-100) should be sampled from several different geographic areas.

Few studies (Lang et al. 2006, 2007) have attempted to clarify relationships among N. American *Castanea* taxa using cpDNA sequences. Lang et al. (2007) showed that cpDNA sequences can be used to successfully study systematic relationships within *Castanea*. They sampled 18 accessions of seven *Castanea* species and used cpDNA from the *trnT-L-F*, *ndhF*, *ycf6-psbM*, *ycf9-trnGM*, and *rpl16* regions to show that *C. ozarkensis* was sister to *C. dentata* plus *C. pumila*. Chloroplast DNA diversity in this genus was highly structured, with haplotypes delineated to a specific geographic area. They identified seven haplotypes; one haplotype circumscribed to *C. ozarkensis* was found in *C. pumila* from Virginia. Lang et al. (2007) attributed this to a complex biogeographical history for *Castanea*, with the possibility of several colonization routes post-glaciation. To our knowledge, this is the only published molecular phylogeny of N. American *Castanea*; however, sampling was limited in number (n= 2 for *C. dentata*, n= 2 for *C.*

ozarkensis, n= 6 for *C. pumila*) and in range (no accessions from morphologically confounding populations in Tennessee or Georgia). Recent work (Sewell et al. 1996; Maskas and Cruzan 2000; Ohi et al. 2003; Shaw and Small 2005; Naciri and Gaudeul 2007; Prentice et al. 2008) has emphasized the importance of obtaining multiple accessions per species because haplotypes may not necessarily be confined to species and can have geographic structuring that may not be revealed without widespread repetitive sampling. Additionally, although the regions thus far sequenced for *Castanea* are popular ones for molecular studies in plants, they have been shown to be relatively slowly evolving noncoding regions of the chloroplast (Shaw et al. 2007). We hypothesized that with the use of a more quickly evolving cpDNA region and with a greater number of accessions from throughout the ranges of the N. American *Castanea* species, we could add significantly to the growing body of molecular evidence that is helping to untangle the evolutionary complexity of N. American *Castanea*. The main goals of the present study were to: 1) determine if genetics reflect the morphological variation observed in southeastern populations of N. American *Castanea*, 2) explore the extent of cpDNA haplotype sharing in N. American *Castanea*, and 3) map haplotype distribution in relation to morphotaxa. Finally, we wanted to use the information obtained from these analyses to gain insight into southeastern populations, where intermediate morphologies and putative hybridization are confounding taxonomy.

Materials and Methods

Taxon sampling and morphological separation of taxa—We obtained *Castanea* accessions from sympatric, allopatric, morphologically intermediate and/or putative hybrid populations of *C. dentata*, *C. pumila*, and/or *C. ozarkensis* between May 2006 and

July 2008. We sampled 234 individuals from populations throughout the ranges of N. American *Castanea*, with much greater sampling in the southern Appalachian Mountains where putative hybrids or morphological intermediates have been identified (Table 1). We sampled as many trees as possible from a population, collected the new growth from each tree from which DNA was extracted, and made a herbarium voucher specimen for each accession; all voucher specimens were deposited at the University of Tennessee at Chattanooga herbarium (UCHT). We immediately stored leaf tissue on ice before long-term storage at 80°C.

We identified *Castanea* species using the following treatments: Radford et al. 1968; Johnson 1988; Wofford 1989; Nixon 1997; Weakley (in prep.). Species calls were made using the following characters: ***Habit:*** *C. dentata*: formerly a massive tree to 30 m. now persists mostly as multi-stemmed resprouts to 5-10 m because of the effect of chestnut blight. *C. pumila*: soboliferous or non-soboliferous shrub or small tree, usually 2-5 meters but up to 15 meters tall. *C. ozarkensis*: never soboliferous, large multi-stemmed shrub or medium-sized tree up to 20 meters tall. ***Leaves*** (Fig. 1): *C. dentata*: typically glabrous, 9-30.1 cm X 3.2-10.5 cm; lance-elliptic or lanceolate; apex acuminate to long-acuminate. *C. pumila*: usually puberulent to tomentose, 4.1-21.7 cm X 1.5-8.3 cm; leaf shape is highly variable; apex acute. *C. ozarkensis*: puberulent to tomentose, 4.3-26.6 cm X 2-9.3 cm; leaf shape is variable, tending to be lance-elliptic or lanceolate; apex is frequently acuminate or long-acuminate. *Castanea X neglecta*: essentially glabrous to tomentose, 4.1-30.1 cm X 1.5-10.5 cm; leaf shape is variable, elliptic to lance-elliptic; apex is acuminate to long-acuminate. ***Fruits:*** *C. dentata*: typically three fruits per cupule; fruits are obovate, tending to be flat on at least one side. *C. pumila* and *C. ozarkensis*:

usually a single fruit per cupule, conical in cross-section. *Castanea* X *neglecta*: 1-2 fruits per cupule; conical or obovate in cross-section, depending on the number of fruits. ***Note:*** The leaves of *C. dentata* tend to be larger, thinner, less pubescent, more deeply serrate, more lanceolate, and have a longer and more acuminate apex than those of *C. pumila.* The leaves of *C. ozarkensis* tend to be larger, more frequently lance-elliptic or lanceolate and acuminate or long-acuminate than *C. pumila. Castanea* X *neglecta* has less pubescent leaves with more numerous bulbous trichomes and slightly larger nuts than *C. pumila* (Johnson 1988). *Castanea X neglecta* hybrids are believed to be widespread in the southeast where they are difficult to differentiate from *C. pumila* (Nixon 1997) because of the wide morphological variation attributed to each of these taxa which is complicated overlapping morphological descriptions for *C. pumila* and *C. X neglecta.* It is likely that the morphologically intermediate accessions used in this study are representative of this putative hybrid taxon *C. X neglecta.* We divided the morphologically ambiguous accessions into to two groups: 1) those with 'Pocket' morphology (i.e., *C. dentata* leaf size and shape with distinct ciliate margins yet only having one fruit per cupule, where fruiting material was observed) that are denoted as 'Intermediate* in Table 1, and 2) those with intermediate morphology (a composite group with any accessions that had leaf morphology that combined *C. dentata* and *C. pumila* characteristics but lacked a ciliate margin and had unknown flower/fruit morphology), denoted as 'Intermediate' in Table 1.

Selection of a Molecular Marker—Because previous molecular studies showed low levels of variation among N. American *Castanea* species (Lang et al. 2007), we screened nine of the most quickly evolving noncoding cpDNA regions sensu Shaw et al. (2005, 2007). Rather than sequence accessions of all three *Castanea* species to identify

most quickly evolving cpDNA regions, we took a PCR-based approach to look for a region in which there were size polymorphisms (indels) that may be diagnostic for each of the three currently recognized taxa. To that end, we screened five *Castanea* accessions, one each for the three named species plus one morphological intermediate as well as one with the Pocket morphology, across nine noncoding cpDNA regions (*atp*I-*atp*H, *psb*A-*trn*H, *rpl*32-*trn*L, 3′*trn*V-*ndh*C, *trnS-trnG-trnG*, *psb*E-*pet*L, *trn*Q-5′-*rps*16, *psb*D-*trn*T, *rps*16-5′*trn*K). We selected the *trn*V-*ndh*C region for the sequencing portion of this study because it initially provided informative length variation among *C. dentata*, *C. pumila*, and *C. ozarkensis* (Kennedy 2008).

DNA extraction, amplification and sequencing—We extracted DNA from leaves using the DNeasy Plant Mini kit (Qiagen, Valencia, California, USA). We performed the polymerase chain reaction (PCR) using Eppendorf (Westbury, New York, USA) Mastercycler gradient or Mastercycler personal thermal cyclers in 25 μL volumes with the following reaction components: 1 μL template DNA (~10–100 ng), 1× *ExTaq* buffer (PanVera/TaKaRa, Madison, Wisconsin, USA), 200 μmol/L each dNTP, 3.0 mmol/L $MgCl_2$, 0.1 μmol/L each primer, and 1.25 units *ExTaq* (PanVera/TaKaRa). Reactions included bovine serum albumin at a final concentration of 0.2 μg/μL, which is known to improve amplification from difficult templates. PCR and sequencing primers were *trn*$V^{(UAC)}$x2F (5'-GTC TAC GGT TCG ART CCG TA-3') and *ndh*C-R (5'-TAT TAT TAG AAA TGY CCA RAA AAT ATC ATA TTC-3'). The PCR protocol described next was preceded by template DNA denaturation at 80°C for 5 min and followed by a final extension step of 1 hour at 65°C. The PCR cycling conditions were 29 cycles of denaturation at 95°C for 15 sec., primer annealing at 50°C for 15 sec., followed by a

ramp of 0.3°C/s to 65°C, and primer extension at 65°C for 5 min. We checked PCR products on 1% agarose gels before cleaning with ExoSAP-IT (USB, Cleveland, Ohio, USA). In most cases, we performed DNA sequencing using only the reverse primer *ndh*C-R; however, in a two cases, the forward primer was needed. We performed all DNA sequencing with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit, v. 3.1 (Perkin-Elmer/Applied Biosystems, Foster City, California, USA) and sequences were electrophoresed and detected on an ABI Prism 3100 automated sequencer (University of Tennessee Molecular Biology Resource Facility, Knoxville, Tennessee, USA). We used Sequencher 4.7 (Gene Codes, Ann Arbor, Michigan, USA) to edit the DNA strands and check any base calls at variable positions. All unique haplotypes sequences were deposited in GenBank.

Alignments and phylogenetic analyses—We aligned DNA sequences by eye in MacClade v. 4.08 (Sinauer, Sunderland, Massachusetts, USA) and double-checked variable positions in the data matrix against the original chromatogram files to make sure that all base calls were true at all variable positions. In all cases but one, alignment of potentially informative positions was unambiguous. In nearly all of the 234 individual sequences there was a "hiccup" or 40 bp area of ambiguous sequencing between base pairs number 220-260 from the 3′ end of *ndh*C; since this area defied base-calling it was excluded from all analyses as no confident alignment could be constructed. We coded indels as binary characters except in the case of a single poly-A/T run near the *trn*V end of the intergenic spacer that was omitted from the data set because it may be a PCR artifact and not reflective of relationships within the group.

We conducted analysis of phylogenetic relationships using the optimality criterion

of maximum parsimony. We executed searches for most-parsimonious trees in PAUP* v. 4.0b10 (Swofford 2002) by a heuristic search with tree-bisection-reconnection (TBR) branch swapping and 10000 random sequence addition replicates with the "collapse zero-length branches" option in effect. We estimated bootstrap support (Felsenstein, 1985) with 10000 replications of heuristic search and simple taxon addition with the constraint of 10 000 000 rearrangements per replicate. We used both the consistency and retention indices (CI and RI, respectively) to assess the amount of homoplasy present in the data set. We also employed Bayesian analysis as an alternative means of phylogenetic assessment. Bayesian analysis of the data was performed using MrBayes 2.01 (Huelsenback and Ronquist 2001) to generate posterior probability distribution using Markov chain Monte Carlo (MCMC) methods. No a priori assumptions about tree topology were made. The statistical model of DNA substitution F81 (Felsenstein 1981) was selected as the best fitting maximum likelihood model using Mr. Modeltest 1.1b (Nylander 2002), which is a simplified version of MODELTEST 3.06 (Posada and Crandall 1998). The MCMC process was set to run 1 X 10^6 generations with four chains. Burn-in was estimated visually by plotting log-likelihood values in Microsoft Excel to determine the number of generations that had run before likelihood values reached an asymptote. To calculate the posterior probability of each bipartition a 50% majority-rule consensus tree was constructed from the remaining trees using PAUP* v. 4.0b10 (Swofford, 2002). We also inferred relationships among the chloroplast haplotypes using the software TCS v. 1.21 (Clement et al. 2000), which implements a statistical parsimony approach to estimating gene genealogies. We performed TCS as an alternative means of analysis because it can infer ancestral or intermediate haplotypes (as opposed to assuming

that these haplotypes are extinct). For the TCS analysis, we used the same sequence alignment that was used in the PAUP* analysis described.

Geographic distribution of haplotypes—To assess the distribution of haplotypes within each of the putative taxa of *Castanea* we created distribution maps using range information from the online available USGS range map shape files of Little (1971, 1976, 1977) (http://esp.cr.usgs.gov/data/atlas/little/). We created distribution maps in ArcGIS 9.2 for each of our haplotypes.

Results

trnV-ndhC analysis and inference of chloro-haplotypes—The 3′*trnV-ndh*C intergenic spacer is found within the LSC (Large Single Copy) region of the chloroplast. A GenBank search showed that only one study (Takahashi et al. 2005) had previously used this region. This region has an average length in angiosperms of 1146 bp, ranging from 318-1800 bp, and it is particularly prone to large indels (Shaw et al. 2007). In N. American *Castanea* this region was approximately 515 bp long. Because of a poly-A/T run approximately 120 bp from the *trn*V end, the aligned data set consisted of 390 bp from the *ndh*C end of the intergenic spacer. Within the aligned matrix were seven parsimony informative characters (including three multi-base indels coded as binary characters) and fourteen variable but parsimony uninformative characters. In a 75% majority rule analysis, a heuristic search found eight equally parsimonious trees of 21 steps each with high consistency and retention indices, 1.0 and 1.0, respectively. A phylogeny of the haplotypes found in N. American *Castanea* is shown in the consensus tree (Fig. 2) and bootstrap values are shown above the branches in that figure. The topology of the Bayesian phylogeny agrees with the one generated using parsimony and

the posterior probabilities are shown below the branches in Fig. 2.

The TCS analysis revealed 13 unique haplotypes (Fig. 3 and Table 1) in 233 accessions of N. American *Castanea* that were aggregated into four main haplotype groups (Figs. 2 and 3). Haplotype group D was found in 63 accessions of *C. dentata* from the historical range of American chestnut and includes haplotypes 2 and 13. Haplotype 2 was found in 57 *C. dentata* accessions from Maine to Georgia. Haplotype 13 was found in accessions from Tennessee (n=2), North Carolina (n=2), Virginia (n=1), and Pennsylvania (n=1) that also had *C. dentata* morphology.

Haplotype group P included haplotypes 1 and 9 and was found in 42 accessions from Virginia to Georgia and in Alabama and Tennessee. The majority of haplotype 1 (n=31) exhibited the morphology of *C. pumila*; however, 4 accessions had *C. dentata* morphology. Some southern Appalachian trees from Georgia (n=7) had haplotype 9 and exhibited *C. pumila* morphology.

Haplotype group O was sampled the least (n= 14) and included haplotypes 3, 11, and 12. Haplotype O was mostly restricted to Arkansas and Missouri, although there were disjunct individuals in Virginia and the Florida panhandle. Trees with *C. ozarkensis* morphology (n=10) from Arkansas and Missouri had haplotype 3. Interestingly, two trees from Virginia that are morphologically *C. pumila* also had this haplotype. Haplotype 11 was found in one *C. pumila* sample from the Florida panhandle and haplotype 12 was found in one *C. pumila* from Arkansas.

Haplotype group M was the most numerous haplotype observed; it was found in 114/233 accessions. Haplotype M was concentrated within the southeastern United States (in Louisiana, Mississippi, Alabama, Georgia, Tennessee, North Carolina, and

Virginia); no northern *C. dentata* or western *C. ozarkensis* had this haplotype. Haplotype M contained six lesser haplotypes (4, 5, 6, 7, 8, and 10). Haplotype 4 was the most numerous (n= 63) and geographically widespread, found in both *C. dentata* (n=40) and *C. pumila* (n=12) accessions from Tennessee and Georgia, and also in *C. pumila* accessions from Louisiana (n=1), Mississippi (n=2), and Alabama (n=2) (Fig. 7). Haplotype 5 was found in most trees from the morphologically distinct Pocket population (n=34), except for a single accession that had haplotype 4. The southernmost *C. pumila* samples from southwest Georgia (n=8) had haplotype 6 and the soboliferous *C. pumila* (n=6) from northern Florida had haplotype 8. A single *C. dentata* accession from N. Carolina had the unique haplotype 10. Two accessions from Alabama (one with *C. dentata* and one with intermediate morphology) had haplotype 7.

Discussion

We hypothesized that by using the quickly evolving *trn*V-*ndh*C noncoding chloroplast region to examine a greater number of accessions from throughout the respective ranges of the commonly recognized *Castanea* taxa that we could add considerably to the growing body of molecular data that is helping to untangle the evolutionary complexity of N. American *Castanea*. To this end we generated cpDNA *trn*V-*ndh*C sequences from 233 accessions of the three commonly recognized N. American taxa in addition to several morphologically curious populations including putative hybrid populations of the southern Appalachians. Specifically, the main foci of this inquiry were to: 1) determine if genetics reflect the morphological variation observed in southeastern populations of N. American *Castanea*, 2) explore the extent of cpDNA haplotype sharing in N. American *Castanea*, and 3) map haplotype distribution in relation

to traditionally defined morphotaxa. Finally, we wanted to gain insight into southern Appalachian populations where intermediate morphologies and putative hybridization defy taxonomy.

Comparison with previous studies of N. American Castanea—Lang et al. (2007) identified seven haplotypes within ~6550 bp of sequence data from five cpDNA regions of N. American *Castanea*. We found thirteen haplotypes, within ~515 bp from a single chloroplast region, with more haplotypes found in *C. pumila* (n=7) and *C. dentata* (n=6) than in *C. ozarkensis* (n=1), but this may reflect the limited geographic range of *C. ozarkensis* and our correspondingly smaller sampling and not be indicative of the true relative genetic diversity of this species. We found shared cpDNA polymorphism in *C. dentata* and *C. pumila*, concentrated in the southern Appalachians and morphologically confounding southeastern populations. Our results do not necessarily support previous studies that show *C. dentata* to have a lack of genetic diversity (Villani et al. 1991; Huang et al. 1994; Dane et al. 2003); however, our data do support the RAPD data of Huang et al. (1994) that showed relatively high genetic diversity in southernmost populations (within which we found that *C. dentata* had haplotype D plus haplotypes M_4, M_6, M_7, M_{10}) that declined toward northern populations (where our sampling only revealed one haplotype within *C. dentata* Haplotype D). As *C. ozarkensis* accessions only had Haplotype O_3, we did not find this species to be as genetically diverse as *C. pumila* or more genetically diverse than *C. dentata* as was found by Dane et al. (2003); however, the intriguing patterns of disjunction within haplotype O may represent more widespread ancestral populations. Johnson (1988) proposed that *C. pumila* and *C. ozarkensis* were derived from *C. dentata*, based on the reduction in the number of flowers and cupule

valves. In a study of allozyme divergence of *Castanea* species, Dane et al. (2003) found *C. dentata* to be sister to a poorly resolved clade with the two chinquapin species, in support of the work by Johnson (1988); however, Lang et al. (2007) found *C. ozarkensis* to be sister to a clade with *C. dentata* and *C. pumila* accessions. In light of the conflicting results from past morphological and molecular studies, it is not surprising that phylogeny generated for this study lacks enough resolution to reveal how these three species (four unique haplotypes) are related to one another (Fig. 2 and 3).

Does morphology match genetic data?—For haplotypes O, D, and P, we found that morphology is closely linked with genetic data the majority of the time (i.e., for 106 of the 114 accessions with these haplotypes, morphology matched genetics). For example, haplotype D was only found in accessions with classic *C. dentata* morphology (Fig. 1b) in individuals from Maine to Georgia. All accessions of *C. ozarkensis* (Fig. 1a) had haplotype O, although three out of the 76 total *C. pumila* accessions also had this haplotype. The majority of accessions with haplotype P had the relatively small leaves with dense white pubescence of the classic *C. pumila* morphology (Fig. 1d), although it is notable that three accessions of southeastern *C. dentata* had this haplotype as well. While cpDNA genetics largely appear to follow morphology for haplotypes O, D, and P, we found abundant morphological diversity within our most numerous and biologically intriguing haplotype M. Within haplotype M, accessions had classic *C. dentata* and *C. pumila* morphology, morphology intermediate between *C. dentata* and *C. pumila* (possibly *C. x neglecta*) (Fig. 1c), 'Pocket' morphology (Fig. 1f), alternative *C. pumila* morphology (Fig. 1e), and the southern soboliferous *C. pumila* morphology (which has leaf morphology like Fig. 1d). It is important to note that although many accessions with

haplotype M do have an intermediate morphology (n = 38) that would be expected to result from hybridization and introgression, a similar amount maintain the characteristic *C. dentata* (n= 44) or *C. pumila* (n= 30) morphology. Interestingly, within haplotype M we found that some populations with unique morphologies formed discrete genetic groups, e.g., the Pocket population that had leaf morphology similar to *C. dentata* but floral and fruit morphology of *C. pumila* also had a unique haplotype (M_5). Additionally, the population of soboliferous *C. pumila* from Florida had its own haplotype (M_8). Trees with the soboliferous morphotype had been treated historically as a separate species, *C. alnifolia*, although Johnson (1988) attributed much of the morphological variation seen in southeastern *C. pumila* as an artifact of ecotypic variation. The sheer number and distribution of chloroplast haplotypes found in this species (n = 7) implies that there is a genetic basis for the observed morphological plasticity. Regardless of whether or not genetically unique groups should receive separate taxonomic treatment, the importance of regional and local haplotypes should be considered when dealing with a species like *C. pumila*.

Interspecific chloroplast haplotype sharing— The fact that we found haplotype M in many of our accessions (n= 114) and that this haplotype group encompasses a variety of morphologies is intriguing. Recently, Lang et al. (2007) reported some cpDNA haplotype sharing among the 10 samples of the three N. American *Castanea* species (a haplotype associated with *C. ozarkensis* was found in two accessions of *C. pumila* from Virginia). Our results corroborate these earlier reports of cpDNA sharing amongst N. American *Castanea* taxa, as we also found a haplotype associated with *C. ozarkensis* (Haplotype O) in two accessions of *C. pumila* from Virginia and one accession from near

the Apalachicola River in Florida. We found Haplotype P, mainly a *C. pumila* haplotype, in three accessions of *C. dentata* in the southern Appalachians. Although the sharing of haplotypes O and P is limited, *C. dentata*, *C. pumila*, and the morphological intermediates appear to share haplotype M frequently, particularly where morphotaxonomic boundaries overlap in the southern Appalachian Mountains.

Since it has been shown that reproductive barriers are not absolute between *Castanea* taxa (Johnson 1988, Rutter et al. 1990), especially in the southern Appalachians (as evidenced by the named hybrid *Castanea X neglecta* Dode.), it seems that a hypothesis of chloroplast sharing because of past or present hybridization is warranted. It is also possible that some of the haplotype sharing observed in N. American *Castanea* is due to lineage sorting. This seems like a likely explanation for the limited sharing of haplotypes O and P among geographically separated *Castanea* accessions. While the phylogenetic placement of shared haplotypes can provide support for or evidence against lineage sorting depending on whether haplotypes appear to be ancestral or more recently derived (Manos et al. 1999), the lack of resolution in our phylogeny prevents its utility in this regard. We propose that hybridization and introgression followed by post-glacial migration is the reason for the majority of the sharing of cpDNA haplotype M among *C. dentata* and *C. pumila*. The Wisconsin glacier (max. 18,000-20,000 ya) drove numerous plant species into southern refugia from the Atlantic coast through Florida and the Gulf Coastal region to the Ozarks where warm climates afforded an ideal refuge. This would have provided an opportune period for chloroplast introgression to occur among closely related species, particularly in taxa such as *Castanea*, where low reproductive barriers and poor seed dispersal could have allowed for hybridization as species' ranges changed

in response to glacial retreat.

Hybridization has shaped the genetic diversity of some eastern N. American taxa (Soltis et al. 2006), and similar patterns of polymorphism resulting from refugia-influenced hybridization have been observed in other plant species. Golden and Bain (2000) found the high number of polymorphic *Packera* populations in southwestern Alberta to be an artifact of intermittent gene flow between species that became sympatric when driven into refugia by expanding glaciation. This process may explain our observations in N. American *Castanea* taxa, which are mostly polymorphic in areas of current sympatry. Studies showed that *Quercus* (Manos et al. 1999), *Pinus* (Matos and Schaal 2000), and *Eucalyptus* (McKinnon et al. 2001) exhibit interspecific cpDNA sharing attributed to hybridization followed by introgression. This scenario is concordant with the recognized tendency for *Castanea* species to hybridize naturally (Johnson 1988; Rutter et al. 1990) and the knowledge that cycles of glacial and interglacial periods during the Pleistocene caused Northern Hemisphere plant species to undergo dramatic range constrictions and expansions (Davis 1976) that may have altered the reproductive barriers between species restricted within refugia.

Geographic Structure of Haplotypes—We found that cpDNA haplotypes correlate with specific geographic areas, supporting the results of Lang et al. (2007) that *Castanea* cpDNA diversity is highly geographically structured. Haplotype D (*C. dentata* morphology) was the most widespread haplotype, found from Maine to Georgia, the geographic region traditionally ascribed to American chestnut. Haplotype O was found mostly in *C. ozarkensis* from the Ozark Mountains; however, haplotype O was also found in three eastern accessions of presumed *C. pumila* from Virginia (n=2) and Florida (n=1),

indicating either disjunct patterns within *C. ozarkensis* (not supported morphologically) or a complex biogeographic/evolutionary history with the possibility of multiple colonization routes after glacial events for *C. pumila* (Lang et al. 2007). Haplotype group P was mostly found in accessions of *C. pumila* throughout the southern Appalachian region, although three *C. dentata* accessions of the southern Appalachians had haplotype P. The range of haplotype M appeared to be centered in the southern Appalachians, and it was found in *C. pumila*, *C. dentata*, and all accessions with intermediate morphology.

Chloroplast DNA haplotype patterns can reflect the possibility that individuals with a certain haplotype have direct or indirect selective advantage (Freeman et al. 2001). Haplotype D was only found in *C. dentata*, which can tolerate a wider range of temperatures than *C. pumila* or *C. ozarkensis*, allowing American chestnut to grow across a greater latitudinal range. It is interesting to note that haplotype D was the only haplotype found in the northern portions of *C. dentata*'s range. Exclusively northern haplotypes have been seen in other temperate deciduous trees such as *Acer saccharum*, *Carya ovata*, and *Betula alleghaniensis* (Soltis et al. 2006). This implies that these species had populations further north during glacial times than previously believed (Soltis et al. 2006). Ancestral haplotypes often have more widespread distributions than those that are more derived (Schaal et al. 1998). This would support Johnson's (1988) belief that *C. dentata* is the most ancient lineage among N. American *Castanea*; however, Lang et al. (2007) found *C. ozarkensis* to be sister to a clade containing *C. pumila* and *C. dentata*. Although haplotype O does not appear to be currently widespread, the observed disjunct patterns may represent a more broad ancestral range. It is likely that *Castanea* taxa were much more widely distributed in the Northern Hemisphere during the

Paleocene to Pliocene, as the fossil record indicates that *Castanea* taxa ranged throughout Greenland, Alaska, Oregon, and Colorado (Sargent 1896). Climate changes were severe in N. America, perhaps resulting in localized extinctions (Guo and Ricklefs 2000). Under these circumstances, a species such as *C. ozarkensis*, which Johnson (1988) believed has less ability to adapt than the more derived *C. pumila*, might have been particularly susceptible to regional extinctions.

The geographic distribution of cpDNA lineages allows for present population patterns to be connected to post-glacial migration routes from separate thermophilic forest refugia. In light of the complex scale, topography, and geography of unglaciated eastern North America (Soltis et al. 2006), it is not surprising to see a complicated phylogeographic pattern in *Castanea*. Various migration routes and refugia have been suggested for N. American *Castanea*. Davis (1976) hypothesized that *Castanea* 'may have survived on the continental shelf or may have moved from its refuge, using the shelf as a migration route and subsequently migrated from east to west as the glacier receded' (p. 22). Davis later revised this to a south-north migration (1983) based on palynological data that indicated the existence of *C. dentata* in the southern Appalachian region 15,000 ya, in the northern Appalachian region 5000 ya, and Connecticut 2000 ya (Delcourt et al. 1980). More recently, Huang et al. (1998) proposed multiple refugia for *Castanea* towards the end of the Wisconsin glaciation, one in southern Alabama and another near the North Carolina or Virginia continental shelf. Regardless of the precise locations *Castanea*'s southern refugia, our results show most of the haplotype diversity was found in populations from southeastern N. America, which support the hypothesis that regions geographically proximate to putative refugia tend to have increased genetic diversity

(Newton et al. 1999).

Soltis et al. (2006) reviewed the phylogeography of unglaciated N. America and noted a series of discontinuities that provide insight into the geographic structure of *Castanea*. Phylogeographic splits that correspond to the Mississippi River are common and this discontinuity appears in *Castanea* as evidenced by *C. ozarkensis*. Haplotype O was mostly found in *C. ozarkensis* accessions west of the Mississippi River, which largely separates Haplotype O from Haplotypes P, D, or M. Haplotype O may have had a refugium in Louisiana or southeast Texas. Haplotype P was found east of the Mississippi River. This central haplotype, distributed in Tennessee, western North Carolina, and northern Alabama and Georgia, that may have had a refugium in southern Alabama or western Florida. There is an additional discontinuity correlated with Apalachicola River, which appears as sub-structuring in the southeastern *Castanea* haplotypes. Overlapping partially with the distribution of Haplotype P but ranging much further north along the Appalachian Mountains is Haplotype D, which may have had a refugium on the continental shelf of North Carolina or Virginia, as proposed by Davis (1976) and Huang et al. (1998). Thus Haplotypes O, P, and D support the possibility of three separate refugia: one west of the Mississippi River (O), and two in the southeast, possibly Alabama (P) and North Carolina (D). Our data add to the growing evidence for one or more refugia in the southern Appalachian Mountains (Soltis et al. 2006). To our knowledge, this pattern of an Apalachicola River discontinuity in addition to a Mississippi River discontinuity has not been observed in plants before although it is fairly common in animals (Soltis et al. 2006). Haplotype M is particularly concentrated in northeastern Alabama, northwestern Georgia and east-central Tennessee. Alabama is

known to be contact zone for closely related species that survived glacial periods in different refugia (Soltis et al. 2006). This region may represent an area of overlapping refugia, where hybridization could have occurred between *Castanea* taxa. Based on spatial analysis of phylogeographic breaks in ten randomly chosen studies of species from unglaciated eastern N. America, Soltis et al. (2006) showed a density of phylogeographic breaks more extreme than expected (based on the null hypothesis that phylogeographic breaks are distributed randomly) that centers on central Tennessee. This is intriguing, as this area correlates to the region of confounding *Castanea* morphology, chloroplast sharing, and it is the center of the range of the perplexing haplotype M (Fig. 6).

Future Directions— Based on our cpDNA data, as well as conflicting results from previous molecular studies, the phylogenetic relationships of N. American *Castanea* taxa are evolutionarily complex and as such remain incompletely resolved. Past cpDNA phylogenies (Lang et al. 2006, 2007) indicate that *Castanea* is a monophyletic genus originating in Asia; however, our cpDNA sequence data (unpublished) show two putative accessions of *Castanea henryi* (Chinese chinquapin) ("putative" because the seeds were obtained by J.H.C. in a Chinese market) sharing haplotype P_9 with southern Appalachian *C. pumila*. Thus, because of the apparently complex biogeographic history of this genus, we believe it would be prudent to re-examine species relationships within all of *Castanea*, including more accessions per taxon and wider geographic sampling to capture rare or potentially shared haplotypes. The precise genetic basis of the confounding morphology in southeastern populations of N. American *Castanea* is still uncertain but appears to be the result of an independent evolutionary lineage that has been

morphotaxonomically recognized as a hybrid (*C. X neglecta*); however an intricate biogeographical history combined with the tendency of taxa within this genus to share haplotypes in sympatric areas could explain much of the morphological complexity of *Castanea*. It is possible that some of the confounding morphology is due to *Castanea* individuals that have the nuclear genotype of the original pollen parent (and corresponding similar morphology) but having the cpDNA genome of the maternal parent (Freeman et al. 2001). Genomic tools such as nuclear markers are currently being developed for Fagaceae (Wheeler and Sederoff 2008), and further *Castanea* research employing these markers may reveal residual nuclear genes gained from hybridization and provide greater understanding of the genetic basis for intriguing morphologies within this genus.

List of References

Avise, J.C. 1994. Molecular Markers, Natural History and Evolution. Chapman & Hall, New York.

Avise, J.C. 2000. Phylogeography. The History and Formation of Species. Harvard University Press, Cambridge, MA.

Camus, A. 1929. *The Chestnuts: Monograph of Castanea and Castanopsis*. Paul Lechevalier. Paris.

Clement, M., D. Posada and K. Crandall. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9(10): 1657-1660

Comes, H.P. and R.J. Abbott. 1999. Population genetic structure and gene flow across arid versus mesic environments: a comparative study of two parapatric *Senecio* species from the near east. *Evolution* 53(1): 36-54.

Daehler, C.C. and D.R. Strong. 1997. Hybridization between introduced smooth cordgrass (*Spartina alterniflora*; Poaceae) and native California cordgrass (*S. foliosa*) in San Francisco Bay, California, USA. *American Journal of Botany* 84 (5): 607-611

Dane, F., L.K. Hawkins, and H. Huang. 1999. Genetic variation and population structure of *Castanea pumila* var. *ozarkensis*. *Journal of the American Society of Horticultural Sciences* 124(6): 666-670.

Dane, F., P. Lang, H. Huang, and Y. Fu. 2003. Intercontinental genetic divergence of *Castanea* species in eastern Asia and eastern North America. *Heredity* 91: 314-321.

Davis, M.B. 1976. Pleistocene biogeography of temperate deciduous forests. *Geoscience and Man* 3: 13-26.

Davis, M.B. 1983. Quaternary history of deciduous forest of eastern North America and Europe. *Annals of the Missouri Botanical Garden* 70: 550-563.

Delcourt, P.A., H.R. Delcourt, R.C. Brister, and L.E. Lackey.1980. Quaternary vegetation history of the Mississippi embayment. *Quaternary Research* 13:111-132.

Devos, N., D. Tyteca, O. Raspe, R. A. Wesselingh, and A.L. Jacquemart. 2003. Patterns of chloroplast diversity among western European *Dactylorhiza* species (Orchidaceae). *Plant Systematics and Evolution* 243: 85-97.

Dumolin-Lapegue, S. B. Demesure, S. Fineschi, V. Le Corre, and R.J. Petit. 1997. Phylogeographic structure of white oaks throughout the European continent. *Genetics* 146:1475-1487.

Felsenstein, J. 1981. Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution* 17: 368-376.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:783-791.

Freeman, J.S., H.D. Jackson, D.A. Steane, G.E. McKinnon, G.W. Dutkowski, B.M. Potts, and R.E. Vaillancourt. 2001. Chloroplast DNA phylogeography of *Eucalyptus globulus*. *Australian Journal of Botany* 49: 585-596.

Fu, Y. and F. Dane. 2003. Allozyme variation in endangered *C. pumila* var. *pumila*. *Annals of Botany* 92: 223-230.

Furini, A. and J. Wunder. 2004. Analysis of eggplant (*Solanum melongena*)-related germplasm: morphological and AFLP data contribute to phylogenetic interpretations and germplasm utilization. *Theoretical and Applied Genetics* 108: 197-208.

Gleason, H.A. and A. Cronquist. 1991. Manual of Vascular Plants of Northeastern United States and Adjacent Canada. The New York Botanical Garden, Bronx, New York.

Golden, J.L. and J.F. Bain. 2000. Phylogeographic patterns and high levels of chloroplast DNA diversity in four *Packera* (Asteraceae) species in southwestern Alberta. *Evolution* 54(5): 1566-1579.

Gonzales, E., J.L. Hamrick, and S. Chang. 2008. Identification of glacial refugia in south eastern North America by phylogeographical analyses of a forest understory plant, *Trillium cuneatum*. *Journal of Biogeography* 35: 844-852.

Guo, Q, and R.E. Ricklefs. 2000. Species richness in plant general disjunct between temperate eastern Asia and North America. *Biological Journal of the Linnean Society* 134: 401-423.

Hillis, D.M. 1987. Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics*18: 23-42.

Huang, H., F. Dane, and J.D. Norton. 1994. Genetic analysis of 11 polymorphic isozyme loci in chestnut and characterization of chestnut cultivars by multi-locus allozyme genotypes. *Journal of the American Society of Horticultural Sciences* 119(4): 840-849.

Huang, H., F. Dane, and T.L. Kubisiak. 1998. Allozyme and RAPD analysis of the genetic diversity and geographic variation in wild populations of the American chestnut. *American Journal of Botany* 85(7): 1013-1021.

Huelsenbeck, J.P. and F. Ronquist. 2001. MrBayes: Baysian inference of phylogenetic

trees. *Bioinformatics* 17: 754-755.

Jakob, S.S. and F.R. Blattner. 2006. A chloroplast genealogy of *Hordeum* (Poaceae): long-term persisting haplotypes, incomplete lineage sorting, regional extinction, and the consequences for phylogenetic inference. *Molecular Biology and Evolution* 23(8): 1602-1612.

Jaynes, R.A. 1964. Interspecific crosses in the genus *Castanea*. *Silvae Genetica* 13: 146-154.

Johnson, G.P. 1988. Revision of *Castanea* Sect. *Balanocastanon* (Fagaceae). *Journal of the Arnold Arboretum* 69: 25-49.

Jones, R.L. 2005. Plant Life of Kentucky. The University Press of Kentucky, Lexington, Kentucky.

Kanno, M., J. Yokoyama, Y. Suyama, M. Ohyama, T. Itoh, and M. Suzuki. 2004. Geographical distribution of two haplotypes of chloroplast DNA in four oak species (*Quercus*) in Japan. *Journal of Plant Research* 117: 311-317.

Kennedy, S. "Chloroplast DNA analysis of putative chestnut chinquapin hybrids" (undergraduate thesis, University of Tennessee-Chattanooga, 2008), 20-22.

Krishnankutty, N. and S. Chandrasekaran. 2008. Linnaeus 300: tips for tinkering morphological taxonomy. *Current Science* 94(5): 565-567.

Kubisiak, T.L., F.V. Hebard, C.D. Nelson, J. Zhang, R. Bernatzky, H. Huang, S.L. Anagnostakis, and R.L. Doudrick. 1997. Molecular mapping of resistance to blight in an interspecific cross in the genus *Castanea*. *Phytopathology* 87: 751-759.

Kubisiak, T.L. and J. Roberds. 2006. Genetic structure of American chestnut populations based on neutral DNA markers. Pages 1009-122 in: Steiner K.C. and J.E. Carlson, eds. Restoration of American Chestnut To Forest Lands-Proceedings of a Conference and Workshop. May 4-6, 2004, The North Carolina Arboretum, Natural Resources Report NPS/NCR/CUE/NRR-2006/001, National Park Service, Washington, DC.

Lang, P., F. Dane, and T.L. Kubisiak. 2006. Phylogeny of *Castanea* (Fagaceae) based on chloroplast *trn*T-L-F sequence data. *Tree Genetics and Genomes* 2: 132-139.

Lang, P., F. Dane, T.L. Kubisiak, and H. Huang. 2007. Molecular evidence for an Asian origin and a unique westward migration of species in the genus *Castanea* via Europe to North America. *Molecular Phylogenetics and Evolution* 43: 49-50.

Les, D.H., M.L. Moody, and S.W.L. Jacobs. 2005. Phylogeny and systematics of

Aponogeton (Aponogetonaceae): the Australian species. *Systematic Botany* 30(3): 503-519.

Lohman, L.G. 2006. Untangling the phylogeny of neotropical lianas (Bignonieae, Bignoniaceae). *American Journal of Botany* 93(2): 304-318.

Makarevitch, I., K. Golovnina, S. Scherbik, and A. Blinov. 2003. Phylogenetic relationships of the Siberian *Iris* species inferred from noncoding chloroplast DNA sequences. *International Journal of Plant Science* 164(2): 229-237.

Manos, P.S., J.J. Doyle, and K.C. Nixon. 1999. Phylogeny, biogeography, and processes of molecular differentiation in *Quercus* subgenus *Quercus* (Fagaceae). *Molecular Phylogenetics and Evolution* 12(3): 333-349.

Maskas, S.D. and M.B. Cruzan. 2000. Patterns of intraspecific diversification in the *Piriqueta carolineana* complex in southeastern North America and the Bahamas. *Evolution* 54(3): 815-827.

Matos, J.A. and B.A. Schaal. 2000. Chloroplast evolution in the *Pinus montezumae* complex: a coalescent approach to hybridization. *Evolution* 54(4): 1218-1233.

McKinnon, G.E., D.A. Steane, B.M. Potts, and R.E. Vaillanocourt. 1999. Incongruence between chloroplast and species phylogenies in *Eucalyptus* subgenus *Monocalyptus* (Myrtaceae). *American Journal of Botany* 86(7): 1038-1046.

McKinnon, G.E., R.E. Vaillancourt, H. D. Jackson, and B.M. Potts. 2001. Chloroplast sharing in the Tasmanian Eucalypts. *Evolution* 55 (4): 703-711.

Morris, A.B. S.M. Ickert-Bond, D.B. Brunson, D.E. Soltis, and P.S. Soltis. 2008. Phylogeographical structure and temporal complexity in American sweetgum (*Liquidambar styraciflua*; Altingiaceae). *Molecular Ecology* 17: 3889-3900.

Myers, B.R., J.L. Walck, and K.E. Blum. 2004. Vegetation change in a former chestnut stand on the Cumberland Plateau of Tennessee during an 80-year period (1921-2000). *Castanea* 69(2): 81–91.

Naciri, Y. and M. Gaudeul. 2007. Phylogeography of the endangered *Eryngium alpinum* l. (Apiaceae) in the European Alps. *Molecular Ecology* 16: 2721-2733.

Newton A.C., T.R. Allnutt, A.C.M. Gillies, A.J. Lowe, and R.A. Ennos. 1999. Molecular phylogeography, intraspecific variation, and the conservation of tree species. *Tree* 14(4): 140-145.

Nixon, K. C. 1997. Flora of North America Editorial Committee (Eds.), Flora of North America North of Mexico, Vol 3. Oxford University Press, NY, pp. 436-437.

Nixon, K.C. and W.L. Crepet. 1989. *Trigonobalanus* (Fagaceae): Taxonomic status and phylogenetic relationships. *American Journal of Botany* 76: 828–841.

Nylander, J.A.A. 2002. Testing models of evolution—MrModeltest 1.1b. Computer program and documentation distributed by author, website: http://www.ebc.uu.se/systzoo/staff/nylander.html.

Ohi, T., T. Kajita, and J. Murata. 2003. Distinct geographic structure as evidenced by chloroplast DNA haplotypes and ploidy level in Japanese *Aucuba* (Aucubaceae). *American Journal of Botany* 90(11): 1645-1652.

Oja, T. and V. Jaaska. 1998. Allozyme diversity and phylogenetic relationships among diploid annual bromes (Bromus, Poaceae). *Annales Botanici Fennici* 35: 123-130.

Posada, D. and K.A. Krandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14: 817-818.

Prentice, H.C., J.U. Malm, and L. Hathaway. 2008. Chloroplast DNA variation in the European herb *Silene dioica* (red campion): postglacial migration and interspecific introgression. *Plant Systematics and Evolution* 272: 23-27.

Radford, A.E., H.E. Ahles, and C. R. Bell. 1968. Manual of the Vascular Flora of the Carolinas. University of North Carolina Press, Chapel Hill, North Carolina.

Rehder, A. 1940. Manual of Cultivated Trees and Shrubs. MacMillian Company, New York, New York.

Robinson, B.L., and M.L. Fernald. 1908. Gray's New Manual of Botany, 7th Edition. American Book Company, New York, Cincinnati, and Chicago.

Rossetto, M. 2005. A simple molecular approach for identifying a rare *Acronychia* (Rutaceae) provides new insights on its multiple hybrid origins. *Biological Conservation* 121(1): 35-43.

Rutter, P.A., G. Miller, and J.A. Payne. 1990. Chestnut. In J.N. Moore, and J.R. Ballington, Jr. [eds.], Genetic resource for temperate fruit and nut crops, 761-788. The International Society for Horticultural Science, Wageningen, The Netherlands.

Sargent, C.S. 1896. Silva of North America 9:10.

Sargent, C.S. 1922. Manual of the Trees of North America. The Riverside Press, Cambridge, Massachusetts.

Schaal, B.A., D.A. Hayworth, K.M. Olsen, J.T. Rauscher, and W.A. Smith. 1998. Phylogeographic studies in plants: problems and prospects. *Molecular Ecology* 7:

465-474.

Sewell, M.M., C.R. Parks, and M.W. Chase. 1996. Intraspecific chloroplast DNA variation and biogeography of North American *Liriodendron* L. (Magnoliaceae). *Evolution* 50(3): 1147-1154.

Shaw, J., E.B. Lickey, J.T. Beck, S.B. Farmer, W. Liu, J. Miller, K.C. Siripun, C.T. Winder, E.E. Schilling, and R. Small. 2005. The tortoise and the hare II: relative utility of 21 noncoding chloroplast DNA sequences for phylogenetic analysis. *American Journal of Botany* 92(1): 144-166.

Shaw, J., E.B. Lickey, E.E. Schilling, and R. Small. 2007. The tortoise and the hare III: comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms. *American Journal of Botany* 92(1):142-166.

Shaw, J. and R.L. Small. 2005. Chloroplast DNA phylogeny and phylogeography of the North American plums (*Prunus* subgenus *Prunus* section *Prunocerasus*, Rosaceae). *American Journal of Botany* 92(12): 2011-2030.

Small, J.K. 1903. Flora of the Southeastern United States. Published by Author, New York, New York.

Small, J.K. 1933. Manual of the Southeastern Flora. Published by Author, New York, New York.

Soltis, D.E., P.S. Soltis, and B.M. Milligan. 1992. Intraspecific chloroplast DNA variation: systematic and phylogenetic implications. Pp. 117-150 in P.S. Soltis, D.E. Soltis, and J.J. Doyle, eds. *Molecular Systematics of Plants*. Chapman and Hall, New York.

Soltis D.E., M.A. Gitzendanner, D.D. Strenge, and P.E. Soltis. 1997. Chloroplast DNA intraspecific phylogeography of plants from the Pacific Northwest of North America. *Plant Systematics and Evolution* 206: 353-373.

Soltis, D.E., A.B. Morris, J.S. McLachlan, P.S. Manos, and P.S. Soltis. 2006. Comparative phylogeography of unglaciated eastern North America. *Molecular Ecology* 15: 4261-4293.

Swofford, D.L. 2002. PAUP*: phylogenetic analyses using parsimony (*and other methods) version 4.0b10. Sinauer Associates, Sunderland, Massachusetts, USA.

Takahashi, S., T. Furukawa, T. Asano, Y. Terajima, H. Shimada, A. Sugimoto, and K. Kadowaki. 2005. Very close relationship of the chloroplast genomes among Saccharum species. Theoretical and Applied Genetics 110: 1523–1529.

Valiejo-Roman, C.M., E.I. Terentieva, T.H. Samigullin, M.G. Pimenov, F. Ghahremani, and V. Mozaffarian. 2006. Molecular data (nrlTS-sequencing) reveal relationships among Iranian endemic taxa of the Umbelliferae. *Feddes Repertorium* 117 (5-6): 367-388.

Villani F, M. Pigliucci, S. Benedettelli, and M. Cherubini. 1991. Genetic differentiation among Turkish chestnut (*Castanea sativa* Mill) populations. *Heredity* 86:131–136

Weakley, A. S. 2005. Flora of the Carolinas, Virginia, Georgia, and Surrounding Areas.

Wheeler, N. and R. Sederoff. 2008. Role of genomics in the potential restoration of the American chestnut. *Tree Genetics & Genomics* DOI 10.1007/s11295-008-0180-y.

Wofford, B.E. 1989. Guide to the Vascular Plants of the Blue Ridge. University of Georgia Press, Athens, Georgia.

Chapter III

Conclusions and Future Directions

The goals of this thesis were to: 1) determine if genetics reflect the morphological variation observed in southeastern populations of N. American *Castanea*, 2) explore the extent of cpDNA haplotype sharing among these taxa, and 3) map haplotype distribution in relation to morphotaxa. I wanted to use the information obtained from these analyses to gain insight into southeastern populations, where intermediate morphologies and putative hybridization confound taxonomy. I hypothesized that with the use of a quickly evolving cpDNA region and with a large number of accessions from throughout the ranges of the N. American *Castanea* species, I could add significantly to the growing body of molecular evidence that is helping to untangle the evolutionary complexity of N. American *Castanea*.

Surprisingly, I found that none of the initial hypotheses was directly supported by our results. It appears that the Pocket population is a separate evolutionary lineage from *C. dentata*, *C. pumila*, and *C. ozarkensis* or any of the Eurasian species, with a unique chloroplast haplotype; however, whether this lineage is what botanists historically have called *C. X neglecta* is likely but uncertain. Limited haplotype sharing exists among N. American *Castanea* species that may be a remnant of past lineage sorting as opposed to current hybridization in sympatric areas. While further research remains to be done before the precise identity of the Pocket trees can be determined, it is clear that the Pocket trees do not share the same haplotype as *C. pumila*, *C. dentata*, *C. ozarkensis* or Asian *Castanea* species.

This research has provided insight into the phylogeographical complexity of N. American *Castanea* and genetic support for the intriguing and confusing morphology observed in southeastern *Castanea* taxa, including the identification of an additional genetic lineage that may previously been called *C. X neglecta*. Broader impacts include the addition of thirteen novel chloroplast DNA sequences that will be added to GenBank, an online genetic sequence database. These sequences can be used to inform future research within this genus. Chapter II of this thesis will be submitted to the peer-reviewed journal *Systematic Botany*. If it is accepted, this paper will add significantly to the growing body of research on the phylogeography of eastern N. America. Furthermore, to my knowledge, this will be the first paper that addresses the phylogeography of an eastern N. American plant genus that employs such an extensive and widespread sampling methodology. Finally, this thesis provides relevant and practical information that can help TACF to identify the most "characteristic" *C. dentata* to target for its breeding program.

Future Directions— The phylogenetic relationships of N. American *Castanea* taxa are evolutionarily complex and as such remain incompletely resolved. Past cpDNA phylogenies (Lang et al. 2006, 2007) indicate that *Castanea* is a monophyletic genus originating in Asia; however, my cpDNA sequence data (unpublished) show two putative accessions of *Castanea henryi* (Chinese chinquapin) ("putative" because the seeds were obtained by J.H.C. in a Chinese market) sharing haplotype P_9 with southern Appalachian *C. pumila*. Thus, because of the apparently complex biogeographic history of this genus, I believe it would be prudent to re-examine species relationships within all of *Castanea*, including more accessions per taxon and wider geographic sampling to capture rare or

potentially shared haplotypes. Also, the precise genetic basis of the confounding morphology in southeastern populations of N. American *Castanea* is still uncertain but appears to be the result of an independent evolutionary lineage that has been morphotaxonomically recognized as a hybrid (*C. X neglecta*). An intricate biogeographical history combined with the tendency of taxa within this genus to share haplotypes in sympatric areas could explain much of the morphological complexity of *Castanea*. It is possible that some of the confounding morphology is due to *Castanea* individuals that have the nuclear genotype of the original pollen parent (and corresponding similar morphology) but having the cpDNA genome of the maternal parent (Freeman et al. 2001). Genomic tools such as nuclear markers are currently being developed for Fagaceae (Wheeler and Sederoff 2008), and further *Castanea* research employing these markers may reveal residual nuclear genes gained from hybridization and provide greater understanding of the genetic basis for intriguing morphologies within this genus. Frozen in J. Shaw's lab at the University of Tennessee at Chattanooga are ~275 extracted DNAs and ~400 leaf tissues from *Castanea* taxa collected throughout the N. American range of this genus. This is a tremendous resource for future students to use in molecular and morphological inquiries into N. American *Castanea*.

List of References

Freeman, J.S., H.D. Jackson, D.A. Steane, G.E. McKinnon, G.W. Dutkowski, B.M. Potts, R.E. Vaillancourt. 2001. Chloroplast DNA phylogeography of *Eucalyptus globulus*. *Australian Journal of Botany* 49: 585-596.

Lang, P., F. Dane, T.L. Kubisiak. 2006. Phylogeny of *Castanea* (Fagaceae) based on chloroplast *trn*T-L-F sequence data. *Tree Genetics and Genomes* 2: 132-139.

Lang, P., F. Dane, T.L. Kubisiak, H. Huang. 2007. Molecular evidence for an Asian origin and a unique westward migration of species in the genus *Castanea* via Europe to North America. *Molecular Phylogenetics and Evolution* 43, 49-50.

Wheeler, N. and R. Sederoff. 2008. Role of genomics in the potential restoration of the American chestnut. *Tree Genetics & Genomics* DOI 10.1007/s11295-008-0180-y.

Haplotype	Morphology	Collector	Number	Year	Herbarium	State	County
O3	ozarkensis	*S. Bost*	324	2006	UCHT	MO	Barry
M6	pumila	*J. Schibig, S. Bost, E. Camp & P. Bartley*	325	2006	UCHT	AL	Blount
M4	pumila	*S. Bost*	326	2006	UCHT	LA	Claiborne
O3	ozarkensis	*S. Bost*	327	2006	UCHT	MO	Barry
O12	pumila	*S. Bost*	328	2006	UCHT	AR	Clark
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	401	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	403	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	404	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	405	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	406	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	407	2007	UCHT	GA	Walker
M4	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	409	2007	UCHT	GA	Floyd
M4	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	410	2007	UCHT	GA	Floyd
M4	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	411	2007	UCHT	GA	Floyd
M5	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	412	2007	UCHT	GA	Floyd
M5	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	413	2007	UCHT	GA	Floyd
M5	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	414	2007	UCHT	GA	Floyd
M5	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	415	2007	UCHT	GA	Floyd
M5	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	416	2007	UCHT	GA	Floyd
M5	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	417	2007	UCHT	GA	Floyd
M5	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	418	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	419	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	420	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	421	2007	UCHT	GA	Floyd
M4	intermediate	*H. Craddock, M. Binkley, & S. Thomas*	422	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	423	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	424	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	425	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	426	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	427	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	428	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	429	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	430	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	431	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	432	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	433	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	434	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	435	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	436	2007	UCHT	GA	Floyd

Table 1. List of *Castanea* taxa used in this investigation, haplotypes, sources and voucher numbers.

Haplotype	Morphology	Collector	Number	Year	Herbarium	State	County
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	437	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	438	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	439	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	440	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	441	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	442	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	444	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	445	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	446	2007	UCHT	GA	Floyd
M5	intermediate*	*H. Craddock, M. Binkley, & S. Thomas*	450	2007	UCHT	GA	Floyd
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	456	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	457	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	458	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	459	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	460	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	461	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	462	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	463	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	464	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	465	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	466	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	467	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	468	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	469	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	470	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	471	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	472	2007	UCHT	GA	Walker
M4	dentata	*H. Craddock, M. Binkley, & S. Thomas*	473	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	474	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	475	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	476	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	477	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	478	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	480	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	484	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	486	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	491	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	495	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	496	2007	UCHT	GA	Walker

Table 1. List of *Castanea* taxa used in this investigation, haplotypes, sources and voucher numbers.

Haplotype	Morphology	Collector	Number	Year	Herbarium	State	County
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	497	2007	UCHT	GA	Walker
M4	pumila	*H. Craddock, M. Binkley, & S. Thomas*	499	2007	UCHT	GA	Walker
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	507	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	508	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	509	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	510	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	511	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	512	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	514	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	515	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	516	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	517	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	519	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	520	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	521	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	522	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	524	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	525	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	526	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	527	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	528	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	530	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	531	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	532	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	533	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	534	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	535	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	536	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	537	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	538	2007	UCHT	NC	Cherokee
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	540	2007	UCHT	NC	Cherokee
M10	dentata	*H. Craddock, M. Binkley, & S. Thomas*	545	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	547	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	549	2007	UCHT	NC	Cherokee
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	554	2007	UCHT	TN	Cumberland
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	555	2007	UCHT	TN	Cumberland
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	556	2007	UCHT	TN	Cumberland
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	557	2007	UCHT	TN	Cumberland
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	558	2007	UCHT	TN	Cumberland

Table 1. List of *Castanea* taxa used in this investigation, haplotypes, sources and voucher numbers.

Haplotype	Morphology	Collector	Number	Year	Herbarium	State	County
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	559	2007	UCHT	TN	Cumberland
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	560	2007	UCHT	TN	Cumberland
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	561	2007	UCHT	TN	Cumberland
P1	pumila	*H. Craddock, M. Binkley, & S. Thomas*	562	2007	UCHT	TN	Cumberland
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	576	2007	UCHT	TN	Monroe
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	612	2007	UCHT	TN	Polk
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	613	2007	UCHT	TN	Polk
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	614	2007	UCHT	TN	Polk
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	615	2007	UCHT	TN	Polk
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	616	2007	UCHT	TN	Polk
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	617	2007	UCHT	TN	Polk
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	618	2007	UCHT	TN	Polk
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	619	2007	UCHT	TN	Polk
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	620	2007	UCHT	TN	Polk
D2	dentata	*H. Craddock, M. Binkley, & S. Thomas*	621	2007	UCHT	TN	Polk
P1	pumila	*J. Shaw*	630	2007	UCHT	TN	Fentress
M4	dentata	*J. Shaw*	631	2007	UCHT	TN	Fentress
M4	dentata	*J. Shaw*	632	2007	UCHT	TN	Fentress
D2	dentata	*J. Shaw*	633	2007	UCHT	TN	Fentress
P1	pumila	*J. Shaw*	634	2007	UCHT	TN	Fentress
M4	dentata	*C. Neel*	635	2007	UCHT	TN	Coffee
D13	dentata	*C. Neel*	636	2007	UCHT	TN	Davidson
P1	dentata	*C. Neel*	637	2007	UCHT	TN	Lincoln
M4	intermediate	*C. Neel*	638	2007	UCHT	TN	Bedford
M4	dentata	*C. Neel*	639	2007	UCHT	TN	Bedford
M4	dentata	*S. Huskins*	642	2007	UCHT	TN	Hamilton
D2	dentata	*J. Shaw*	643	2007	UCHT	TN	Scott
D2	dentata	*H. Craddock*	645	2007	UCHT	GA	Pickens
P1	pumila	*H. Craddock*	647	2007	UCHT	GA	Pickens
P9	pumila	*H. Craddock*	648	2007	UCHT	GA	Pickens
P9	pumila	*H. Craddock*	649	2007	UCHT	GA	Pickens
P9	pumila	*H. Craddock*	650	2007	UCHT	GA	Pickens
P9	pumila	*H. Craddock*	651	2007	UCHT	GA	Pickens
P9	pumila	*H. Craddock*	652	2007	UCHT	GA	Pickens
P9	pumila	*H. Craddock*	653	2007	UCHT	GA	Pickens
D2	dentata	*H. Craddock*	654	2007	UCHT	GA	Pickens
M6	dentata	*H. Craddock*	661	2007	UCHT	TN	Cumberland
P1	pumila	*H. Craddock*	662	2007	UCHT	TN	Cumberland
P1	pumila	*H. Craddock*	663	2007	UCHT	TN	Cumberland

Table 1. List of *Castanea* taxa used in this investigation, haplotypes, sources and voucher numbers.

Haplotype	Morphology	Collector	Number	Year	Herbarium	State	County
D13	intermediate	*H. Craddock*	664	2007	UCHT	TN	Cumberland
P1	pumila	*H. Craddock*	665	2007	UCHT	TN	Cumberland
D2	dentata	*J. Shaw*	666	2008	UCHT	NC	Jackson
D13	dentata	*J. Shaw*	667	2008	UCHT	NC	Jackson
D13	dentata	*J. Shaw*	668	2008	UCHT	NC	Madison
D2	dentata	*S. Fitzsimmons*	669	2008	UCHT	PN	Huntingdon
D2	dentata	*E. Evans*	670	2008	UCHT	ME	Somerset
D2	dentata	*E. Evans*	671	2008	UCHT	ME	Knox
D2	dentata	*E. Evans*	672	2008	UCHT	ME	Piscataquis
D2	dentata	*E. Evans*	673	2008	UCHT	ME	Waldo
D2	dentata	*E. Evans*	674	2008	UCHT	ME	Piscataquis
D2	dentata	*S. Fitzsimmons*	675	2008	UCHT	PN	Westmoreland
D2	dentata	*J. Shaw*	676	2008	UCHT	TN	Fentress
D2	dentata	*J. Shaw*	677	2008	UCHT	TN	Fentress
P1	pumila	*J. Shaw*	678	2008	UCHT	TN	Fentress
P1	pumila	*J. Shaw*	679	2008	UCHT	TN	Fentress
P1	pumila	*J. Shaw*	680	2008	UCHT	TN	Fentress
D2	dentata	*J. Shaw*	681	2008	UCHT	TN	Fentress
D2	dentata	*J. Shaw*	682	2008	UCHT	TN	Fentress
D2	dentata	*J. Shaw*	683	2008	UCHT	TN	Fentress
D2	dentata	*J. Shaw*	684	2008	UCHT	TN	Fentress
P1	pumila	*J. Shaw*	685	2008	UCHT	TN	Fentress
D2	dentata	*J. Shaw*	686	2008	UCHT	TN	Fentress
M4	dentata	*J. Shaw*	687	2008	UCHT	TN	Fentress
P1	pumila	*J. Shaw*	688	2008	UCHT	TN	Fentress
M4	dentata	*J. Shaw*	689	2008	UCHT	TN	Fentress
P1	pumila	*J. Shaw*	690	2008	UCHT	TN	Fentress
P1	pumila	*J. Shaw*	691	2008	UCHT	TN	Fentress
P1	pumila	*J. Shaw*	692	2008	UCHT	TN	Fentress
M4	dentata	*J. Shaw*	693	2008	UCHT	TN	Fentress
O3	ozarkensis	*S. Mourglia*	694	2008	UCHT	MO	Lawrence
O3	ozarkensis	*S. Mourglia*	695	2008	UCHT	MO	Barry
O3	ozarkensis	*S. Mourglia*	696	2008	UCHT	MO	Barry
O3	ozarkensis	*G. Cormier*	704	2008	UCHT	AR	Sharp
O3	ozarkensis	*G. Cormier*	705	2008	UCHT	AR	Sharp
O3	ozarkensis	*G. Cormier*	706	2008	UCHT	AR	Independence
O3	ozarkensis	*G. Cormier*	707	2008	UCHT	AR	Independence
O3	ozarkensis	*G. Cormier*	708	2008	UCHT	AR	Izard
D2	dentata	*J. E. Wykoff*	709	2008	UCHT	PN	Warren

Table 1. List of *Castanea* taxa used in this investigation, haplotypes, sources and voucher numbers.

Haplotype	Morphology	Collector	Number	Year	Herbarium	State	County
D2	dentata	*J. E. Wykoff*	710	2008	UCHT	PN	Warren
D2	dentata	*J. E. Wykoff*	711	2008	UCHT	PN	Warren
D2	dent X pum	*J. Hill*	712	2008	UCHT	MD	Howard
D2	dent X pum	*J. Hill*	713	2008	UCHT	MD	Howard
M4	dentata	*M. Schulman*	724	2008	UCHT	AL	Jefferson
M6	pumila	*M. Binkley & A. Lundy*	726	2008	UCHT	GA	Baker
M6	pumila	*M. Binkley & A. Lundy*	727	2008	UCHT	GA	Baker
M6	pumila	*M. Binkley & A. Lundy*	728	2008	UCHT	GA	Baker
M6	pumila	*M. Binkley & A. Lundy*	729	2008	UCHT	GA	Baker
M6	pumila	*M. Binkley & A. Lundy*	735	2008	UCHT	GA	Baker
M8	pumila	*M. Binkley & A. Lundy*	736	2008	UCHT	FL	Leon
M8	pumila	*M. Binkley & A. Lundy*	738	2008	UCHT	FL	Leon
M8	pumila	*M. Binkley & A. Lundy*	739	2008	UCHT	FL	Leon
M8	pumila	*M. Binkley & A. Lundy*	744	2008	UCHT	FL	Leon
M8	pumila	*M. Binkley & A. Lundy*	749	2008	UCHT	FL	Leon
M8	pumila	*M. Binkley & A. Lundy*	750	2008	UCHT	FL	Leon
O11	pumila	*M. Binkley & A. Lundy*	751	2008	UCHT	FL	Leon
D13	dentata	*J. Flag*	755	2008	UCHT	PN	Wayne
D13	dentata	*H. Craddock*	756	2008	UCHT	VA	Washington
O3	pumila	*H. Craddock*	757	2008	UCHT	VA	Washington
O3	pumila	*H. Craddock*	758	2008	UCHT	VA	Washington
P1	dentata	*E. Lickey*	759	2008	UCHT	VA	Augusta
P1	dentata	*E. Lickey*	760	2008	UCHT	VA	Augusta
M4	pumila	*W. Barger*	761	2008	UCHT	AL	Baldwin
P1	dentata	*D. Morris*	765	2008	UCHT	AL	Morgan
M7	dentata	*L. Brasher*	767	2008	UCHT	AL	Etowah
M6	intermediate	*L. Brasher*	769	2008	UCHT	AL	Etowah
M7	dentata	*L. Brasher*	770	2008	UCHT	AL	Etowah
M4	pumila	*L. Brasher*	771	2008	UCHT	AL	Etowah
M4	pumila	*S. Bost*	774	2006	UCHT	MS	Hinds
M4	pumila	*S. Bost*	775	2006	UCHT	MS	Tishomingo
M4	dentata	*C. Neel*	1000	2007	UCHT	TN	Grundy
M4	dentata	*C. Neel*	1001	2007	UCHT	TN	Grundy
M4	dentata	*C. Neel*	1002	2007	UCHT	TN	Grundy
M4	dentata	*C. Neel*	1003	2007	UCHT	TN	Grundy
M4	dentata	*C. Neel*	1004	2007	UCHT	TN	Grundy
M4	dentata	*C. Neel*	1005	2007	UCHT	TN	Grundy
M4	dentata	*C. Neel*	1006	2007	UCHT	TN	Grundy

Table 1. List of *Castanea* taxa used in this investigation, haplotypes, sources and voucher numbers.

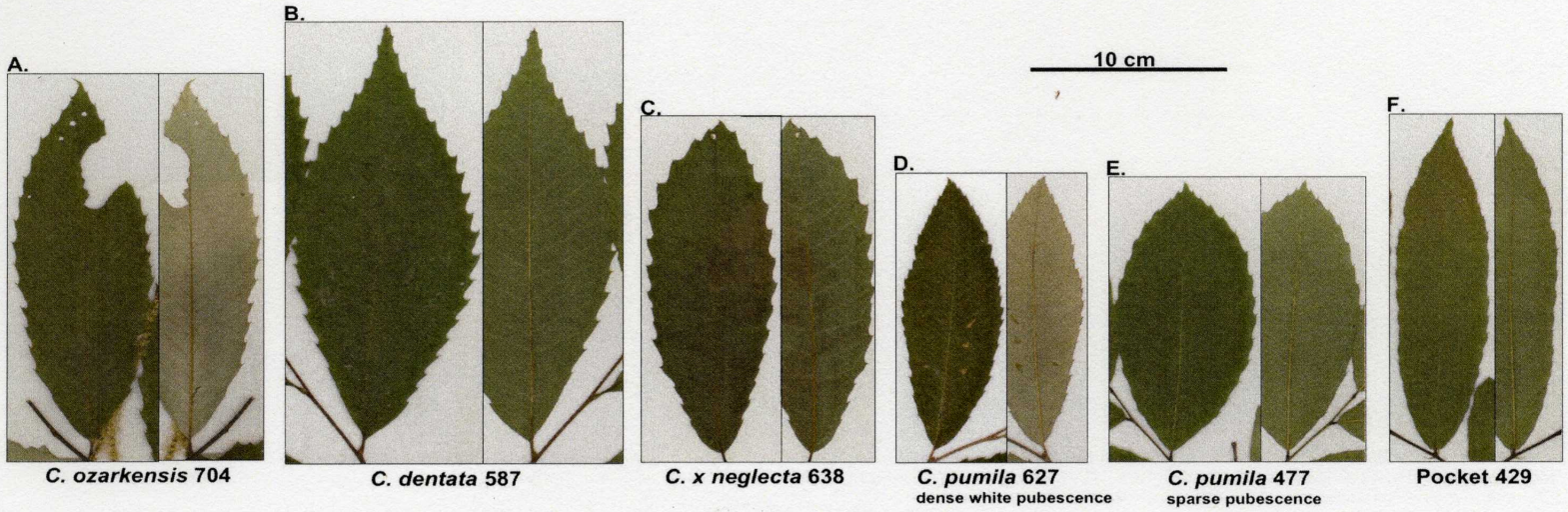

Fig. 1. Representative accessions of North American *Castanea* morphotypes: a) *C. ozarkensis*, b) *C. dentata*, c) Intermediate morphotype, d) *C. pumila,* morphotype 1, e) *C. pumila*, morphotype 2, f) 'Pocket' morphotype

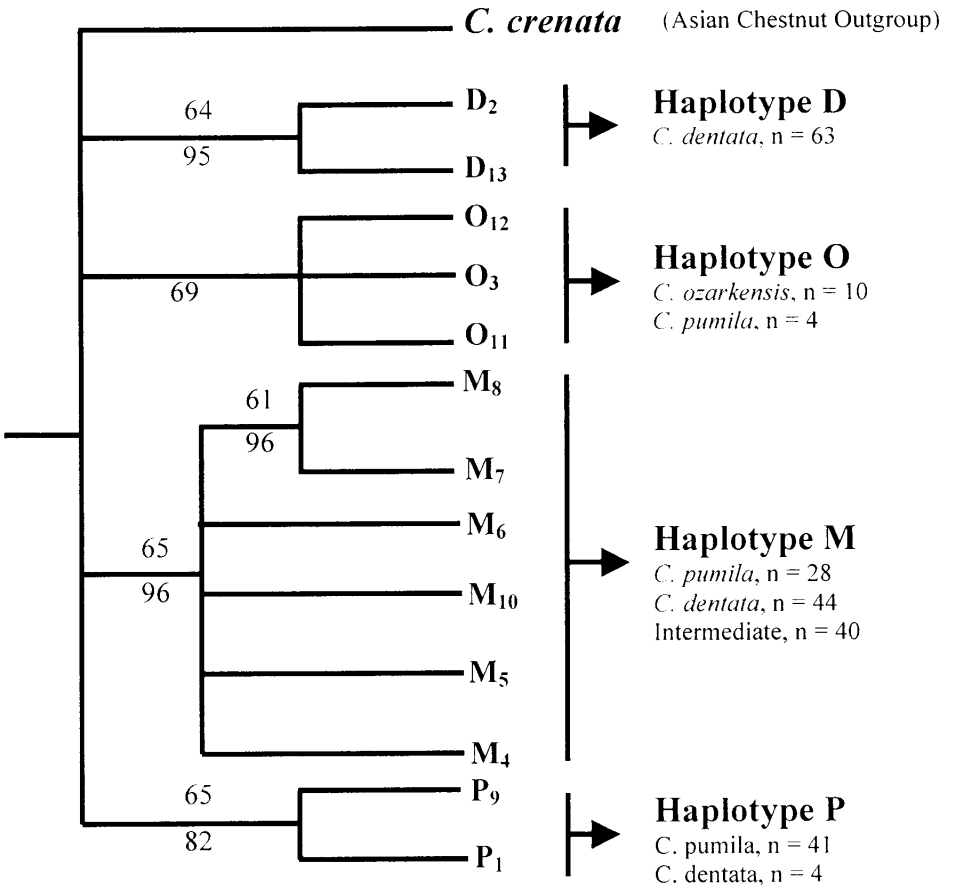

Fig. 2. Consensus tree of 8 equally parsimonious trees resulting from 75% majority rule analysis using the *trn*V-*ndh*C noncoding chloroplast region, showing the relationships of the 13 haplotypes generated from 233 accessions of North American *Castanea* taxa. Parsimony bootstrap values > 50 % are shown above branches. Baysian posterior probabilities > 50 % are shown below branches.

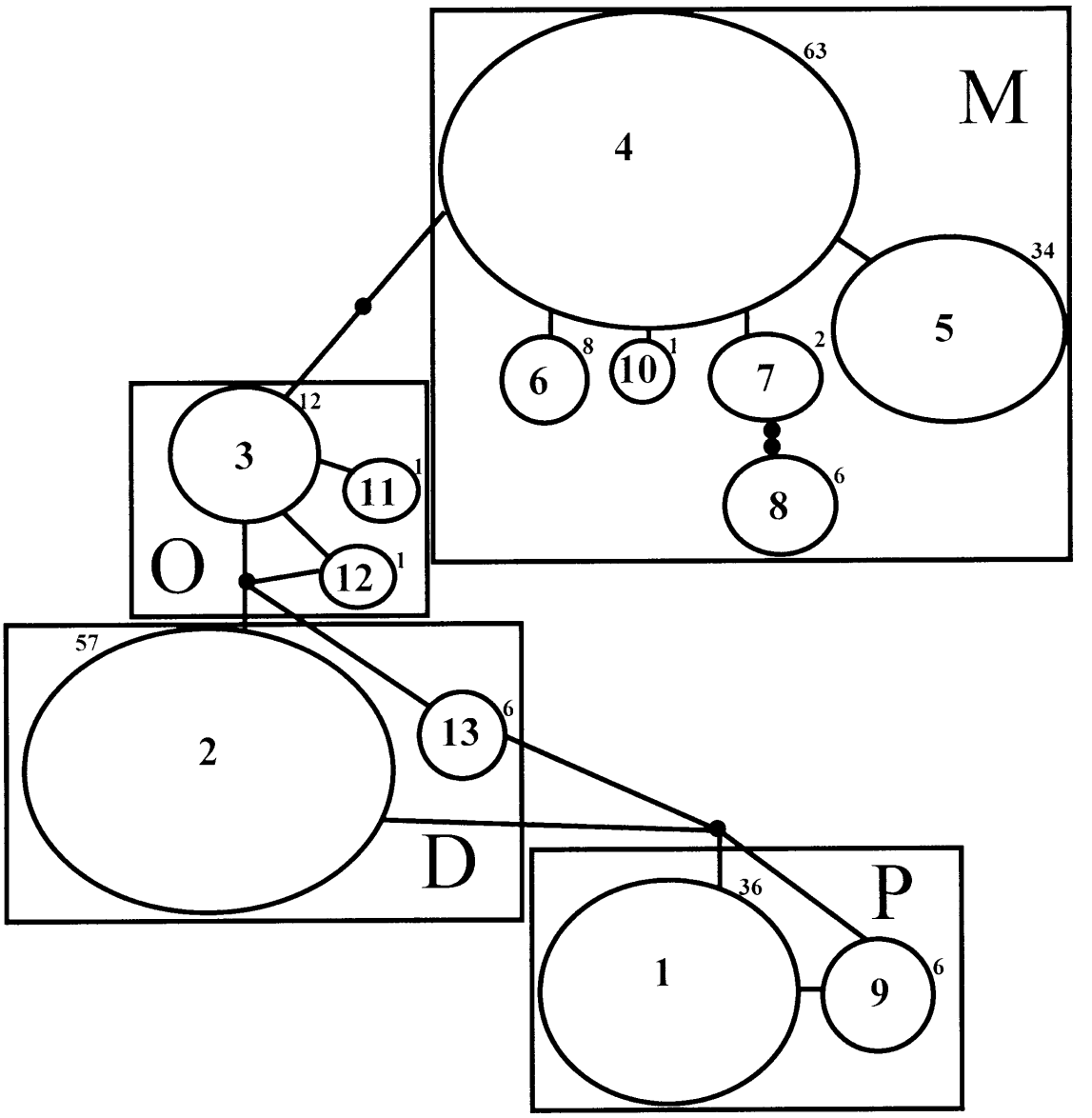

Fig. 3. TCS gene genealogy of *Castanea* taxa from the *trn*V-*ndh*C intergenic spacer region of the chloroplast. Haplotypes observed in this study are represented by circles. The number of times that the haplotype was observed is indicated by the number beside each circle. Lines connecting the haplotypes represent a single mutation (nucleotide substitution or indel) with solid circles representing inferred mutational steps not observed in this study. The 13 haplotypes are grouped into 4 main haplotypes, represented by rectangles. M = mixed morphology, O = *C. ozarkensis* morphology, D = *C. dentata* morphology, P = *C. pumila* morphology.

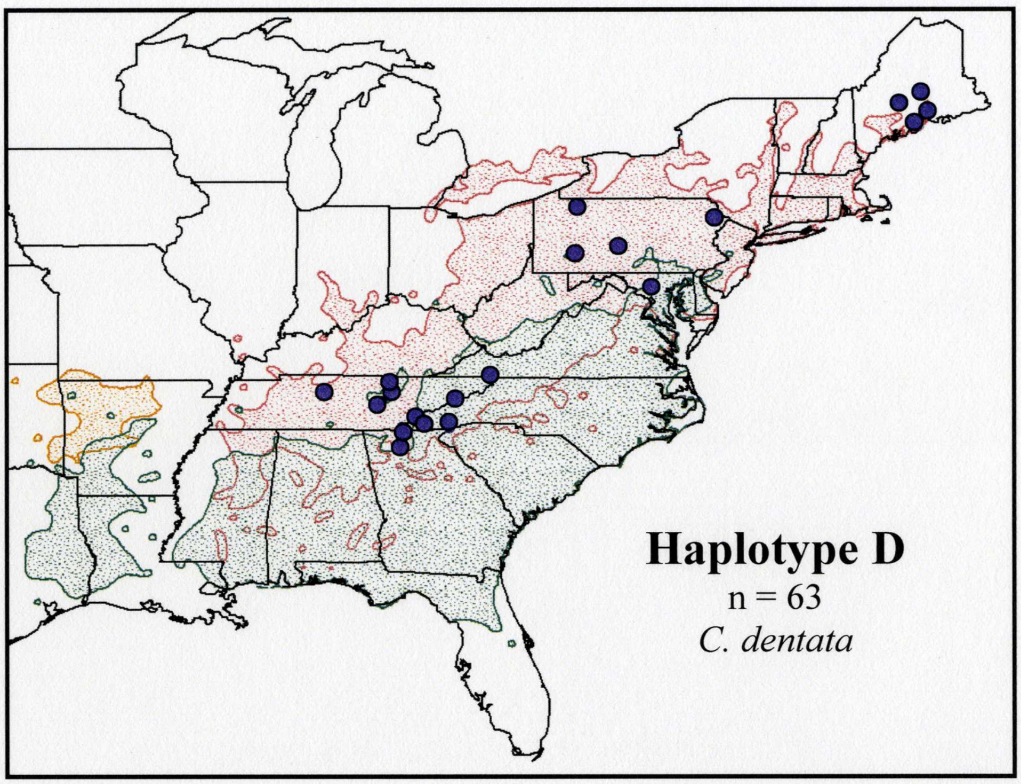

Fig. 4. Map of the geographical range of haplotype D. Circles represent locations of *morphologically C. dentata* accessions.

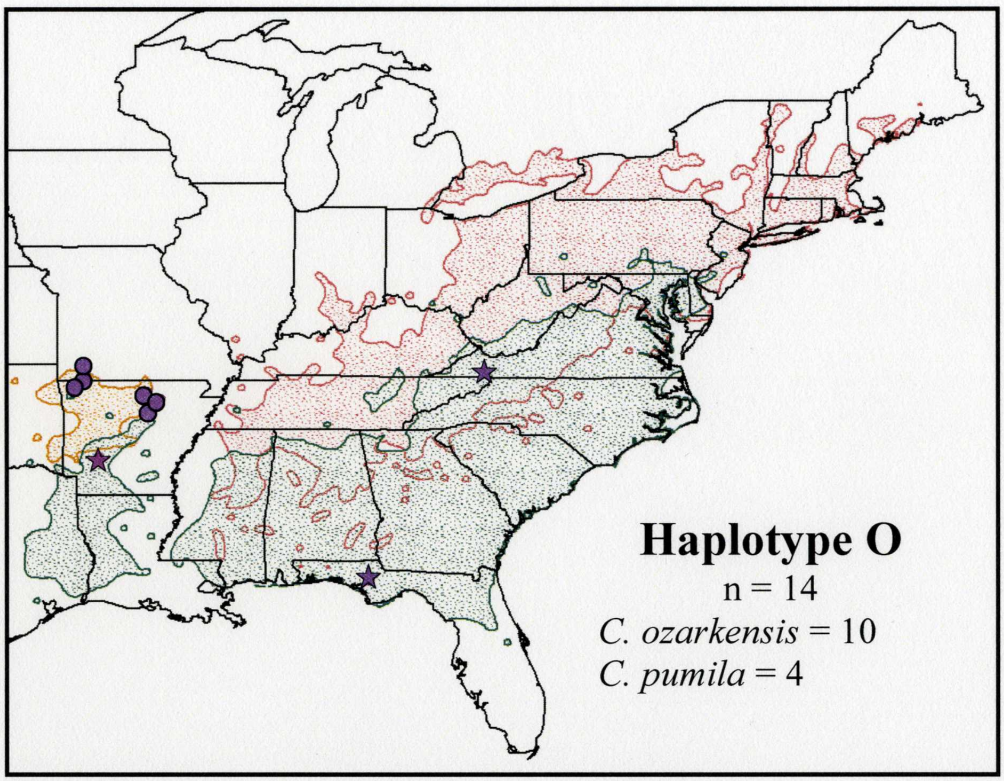

Fig. 5. Map of the geographical range of haplotype O. Circles represent locations of morphologically *C. ozarkensis* populations. Stars represent locations of morphologically *C. pumila* populations.

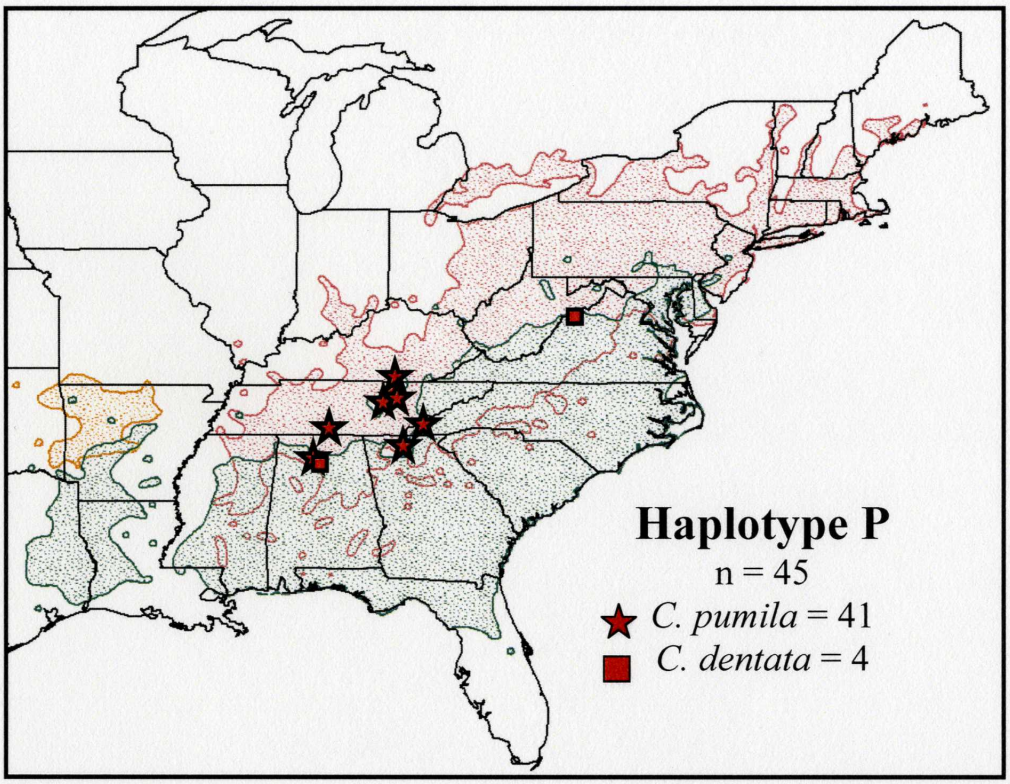

Fig. 6. Map of the geographical range of Haplotype P. Squares represent locations of morphologically *C. dentata* populations, stars represent locations of morphologically *C. pumila* populations.

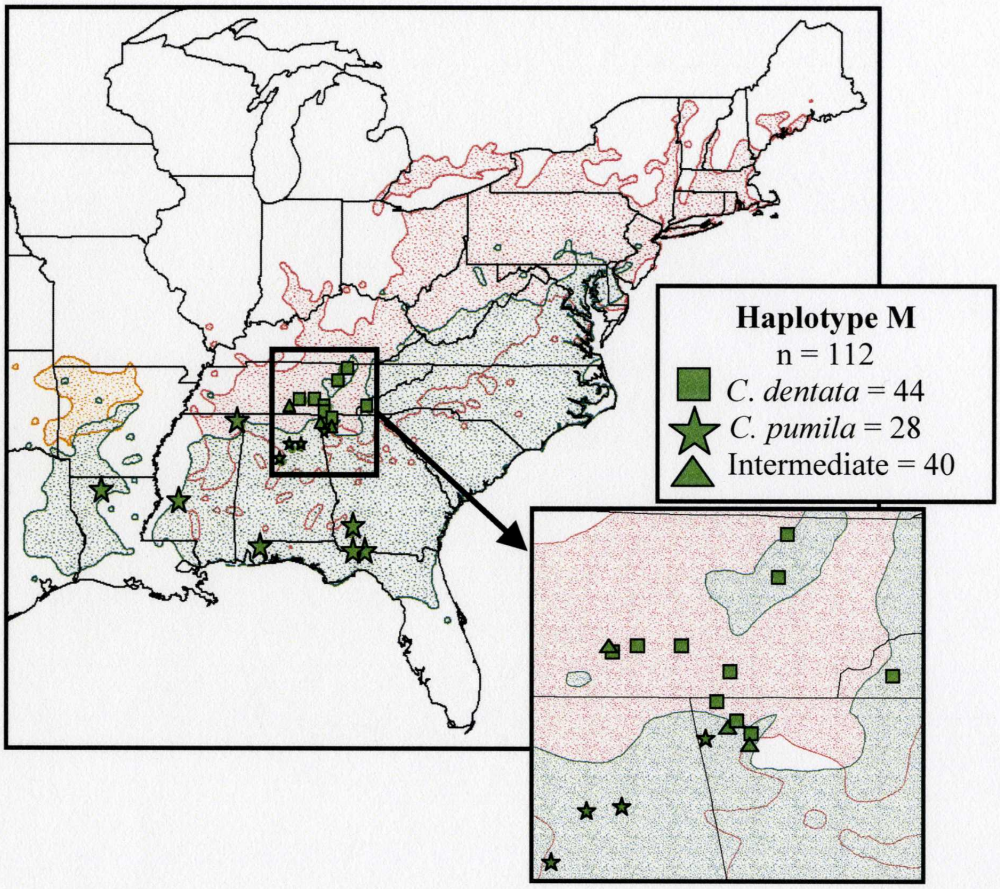

Fig 7. Map of the geographical range of Haplotype M. Squares represent locations of morphologically *C. dentata* populations, stars represent locations of morphologically *C. pumila* populations, and triangles represent morphologically intermediate populations.

Vita

Meagan Binkley was born in Fairfax, Virginia, on September 7, 1983. She grew in Baltimore, Maryland, and Terrell, Texas. She graduated from Terrell High School in Terrell, Texas, in May of 2001. She attended the University of the South, in Sewanee, Tennessee, and received a B.S. in Environmental Studies, magna cum laude with honors in May of 2005. She completed her M.S. in Environmental Science from the University of Tennessee at Chattanooga in December 2008.